THE MECHANISMS AND FUNCTION OF REGULATED IRE1-DEPENDENT DECAY DURING ENDOPLASMIC RETICULUM STRESS

by

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ABSTRACT

The endoplasmic reticulum (ER) is a dynamic organelle that is responsible for the folding and quality control of proteins within the endomembrane system. Both physiological and pathological conditions can result in accumulation of misfolded proteins within the ER, a situation termed ER stress, which results in cell death if not alleviated. Perturbations in ER function result in activation of three ER transmembrane proteins (Ire1, Perk, and Atf6) that are primarily responsible for facilitating the unfolded protein response (UPR). Activation of the UPR initially increases ER capacity to offset the surge in misfolded protein; however, during irremediable stress, the UPR activates pro-apoptotic pathways presumably to prevent the cytotoxic consequences of secreting misfolded proteins.

Ire1 is an endoribonuclease that is responsible for the unconventional splicing of an intron from the transcription factor, Xbp1. However, Ire1 is also responsible for the direct degradation of a number of mRNAs, a process termed regulated Ire1-dependent decay (RIDD). In mammals, long-term activation of Ire1 results in nonspecific cleavage of ER-localized mRNAs and subsequent cell death. However, at early time points a limited number of mRNAs are prioritized to the RIDD pathway and are degraded relatively rapidly.

In the work presented here, I address the questions of (1) how specific mRNAs are prioritized for degradation? And (2) what is the function mRNA degradation during acute of ER stress? I have found that specific nucleotide sequence and structural motifs are used to target mRNAs to the RIDD pathway in both fly and mammalian cells. Furthermore, I show that inhibiting translation of these motifs is also essential for RIDD targeting. Lastly, I show Ire1-dependent effects on lysosome accumulation during ER stress; this may enhance prosurvival signaling of the UPR. These data provide insight into the mechanisms of Ire1 function as well as a model for how the RIDD pathway may function in both the prosurvival and pro-apoptotic pathways of the UPR.

To my parents, Ann and Mike, and my sister, Katy

for their blind support and consistent encouragement

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CHAPTER 1

INTRODUCTION

Overview

Changes to cellular environments, such as the introduction of pathogens or addition of nutrients to a system, require cells not only to process the signals they receive from their environment, but also respond to them appropriately. Many of these responses require secretion of a vast number of proteins. Often, the inability to produce the needed secretion products results in disease states such as diabetes or pathogen infection.

The endoplasmic reticulum (ER) is the entry point for proteins of the secretory pathway, and is responsible for the folding, processing, and quality control of secreted proteins. It is also a highly dynamic organelle that changes its capacity to meet the folding needs of the cell. These changes are often induced through accumulation of unfolded proteins within the ER, which overwhelm the capacity of the organelle to fold proteins efficiently, a situation termed ER stress. Markers of ER stress have been observed in various pathogenic states including cancer and neurological disease (Oakes and Papa, 2015), as well as during physiological processes such as differentiation (Iwakoshi *et al.*, 2003). Thus the ability to understand and manipulate how cells respond to ER stress is thought to provide therapeutic insights into both developmental disorders as well as many diseases.

I sought to determine the mechanism and function of an mRNA degradation pathway induced by ER stress. I first characterize the requirements for mRNA targeting to this pathway in cells from both flies and mammals. I also describe a role for a protein previously thought to function in an entirely separate pathway. Finally, I provide evidence in support of a functional model for how

degradation of a specific mRNA may contribute to the general response to ER stress.

ER Function

Spatial and functional compartmentalization of metabolic and biosynthetic processes is one of the primary distinctions of eukaryotic cells. The endomembrane system consists of the membrane bound organelles that create these compartments. These organelles and vesicles are responsible for transporting proteins from the ER to the plasma membrane (the secretory pathway), as well as for protein degradation and resource recycling, and are essential for cell function (Figure 1.1). In mammalian cells one-third of all proteins are predicted to transit through or remain within the endomembrane system, and in specialized secretory cell types, such as antibody producing plasma cells, hundreds of thousands of proteins are secreted each minute (King and Corley, 1989); therefore, efficiency and maintenance of this system is an essential task.

The ER is a membrane bound organelle responsible for the folding, processing, and quality control of the vast majority of proteins that are part of the endomembrane system. It is also the primary source of lipids for almost all membrane bound organelles within the cell. Thus, it plays an important role in both the structure and function of all other membrane bound organelles. mRNAs that encode endomembrane proteins are translated on ribosomes that dock on the cytosolic side of the ER and translate proteins into the lumen of the ER through the Sec61 channel or translocon (Figure 1.2). These nascent protein chains are then folded into their native conformations with the assistance of ER-

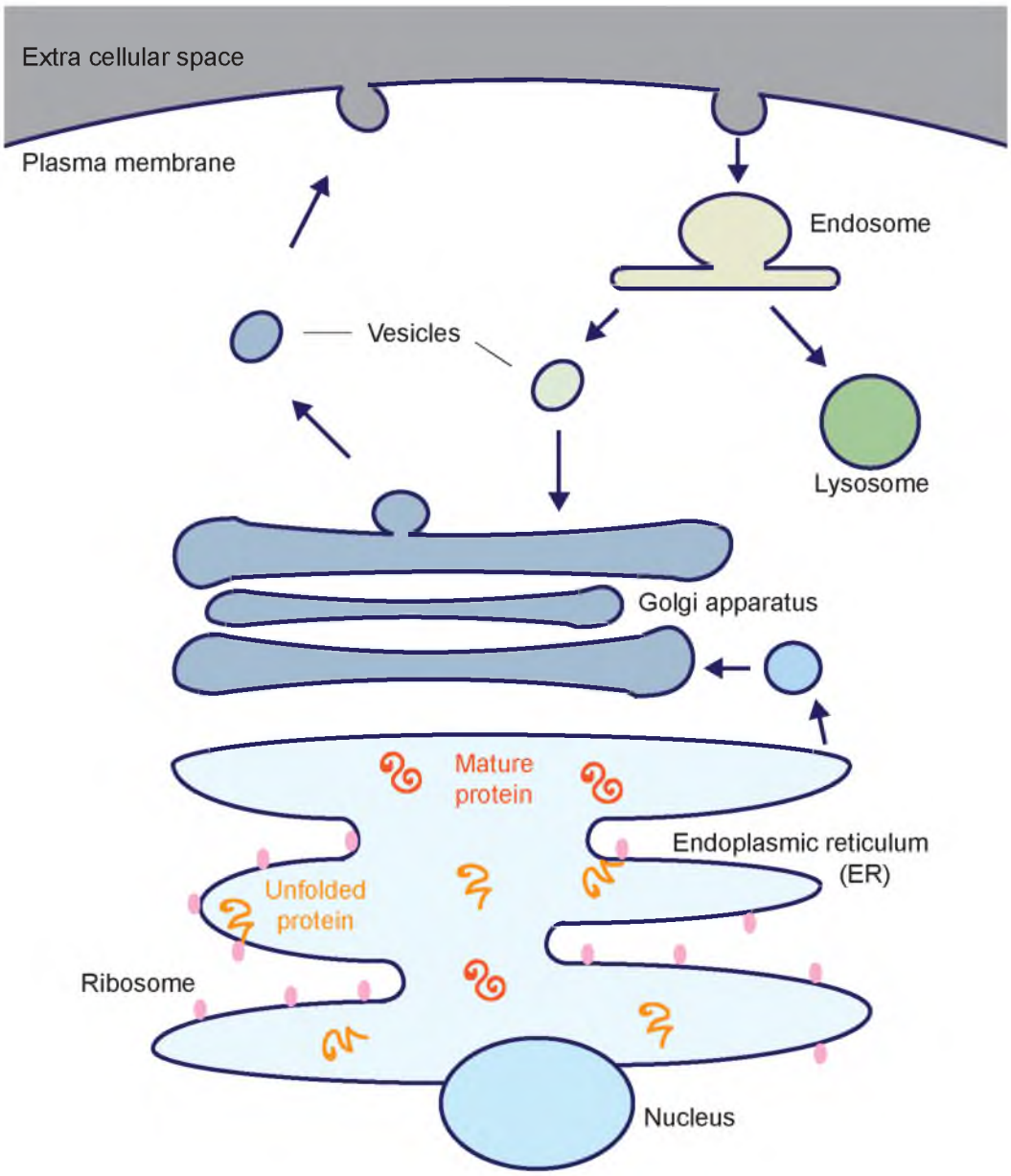


Figure 1.1 The endomembrane system. The endomembrane system is comprised of the nuclear envelope, ER, Golgi apparatus, lysosomes, vesicles, peroxisomes (not pictured), endosomes, and the plasma membrane. Proteins that either remain within the endomembrane system or are secreted out of the cell are translated on ribosomes at the rough ER. Mature proteins exit the ER in vesicles, the majority of which then fuse with the Golgi apparatus, where proteins are further sorted and modified. Proteins leave the Golgi in vesicles destined for other organelles of the endomembrane system. Proteins of the endomembrane system are often cycled through the endosome where damaged proteins are recognized and sent to the lysosome for degradation. (Arrows denote a possible pathway of a transmembrane protein that functions at the plasma membrane.)

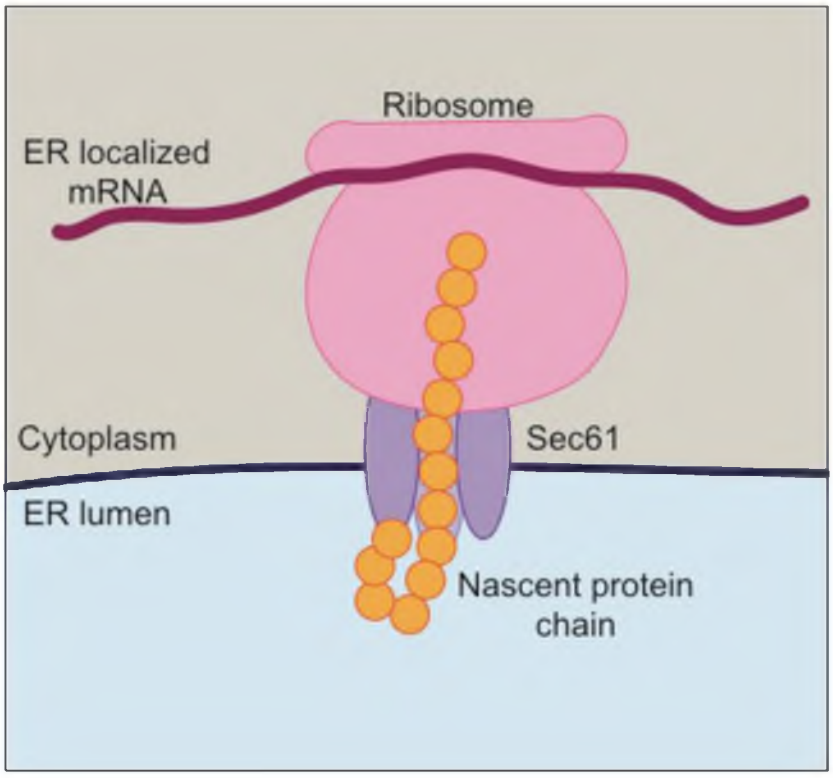


Figure 1.2 Translation of endomembrane proteins at the ER. Proteins containing ER signal sequences or transmembrane domains are co-translationally localized to the ER. Ribosomes dock on the cytosolic side the ER and nascent protein chains are translated through the translocon into the lumen of the ER. During this process the mRNA encoding the protein is stably associated with the cytoplasmic side of the ER through its interactions with the ribosome. This association may be transient or long-term depending on the number of ER-localized ribosomes associated with the mRNA.

resident chaperones. Immature proteins within the ER are also modified through additions such as glycans and disulfide bonds. Once proteins have reached their native conformation, they are sorted and transported out of the ER. To avoid the cytotoxic effects of releasing misfolded proteins, such as aggregation, ER chaperones recognize and sequester proteins that remain terminally misfolded. These proteins are exported back to the cytosol via the ER-associated degradation (ERAD) pathway, where they are degraded in a proteasome-dependent manner (Ruggiano *et al.*, 2014).

ER Stress and the Unfolded Protein Response

The amount of incoming, unfolded proteins is normally balanced with the capacity of the ER to fold and modify them; however, both physiological and pathological events can result in dramatic and rapid increases in the load of proteins entering the ER. ER stress occurs when the number of misfolded proteins within the lumen of the ER overwhelms the ability of the organelle to fold and modify them efficiently. Chemicals that disrupt folding or ER function as well as mutations within hard to fold proteins can also induce ER stress.

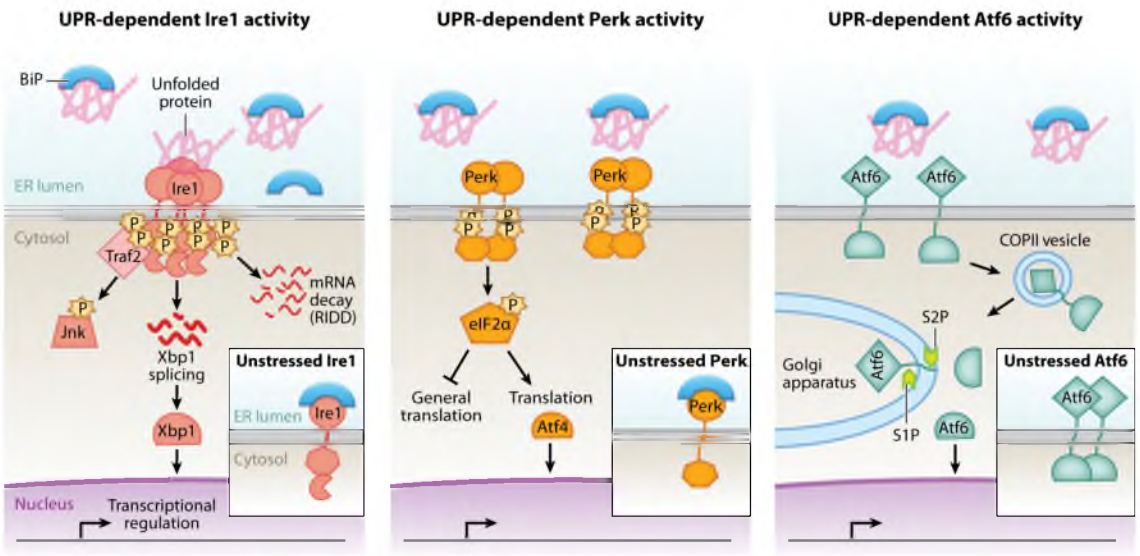
In metazoans, there are three sensors of ER stress: Ire1, Perk, and Atf6. In mammals, there are 2 homologs of both Ire1 and Atf6, noted as -alpha and -beta. These sensors are ER transmembrane proteins that transmit information about the environment of the ER lumen to the cytosol, resulting in various mechanisms that alleviate the folding burden on the ER (Moore and Hollien, 2012). Collectively, these pathways are termed the unfolded protein response (UPR). While the UPR is often considered a response to pathological

accumulation of misfolded proteins, Ire1-alpha and double Atf6-alpha and -beta knockout animals result in embryonic lethality (Urano *et al.*, 2000; Yamamoto *et al.*, 2007). Perk knockout animals are viable, but display severe bone and pancreatic defects, indicating a role for the UPR in development (Zhang *et al.*, 2002). Furthermore, the differences in knockout phenotypes suggest that different branches of the UPR have varied levels of importance depending on cell type and protein-folding burden.

Ire1 is an endoribonuclease that is conserved in eukaryotes. While yeast and flies possess a single Ire1 homolog, mammals possess two homologs of Ire1. Ire1α is ubiquitously expressed (Tirasophon *et al.*, 1998), while Ire1β is confined to intestinal cells (Bertolotti *et al.*, 2001). Plants also have two homologs of Ire1, Ire1A and Ire1B (Koizumi *et al.*, 2001; Noh *et al.*, 2002). Upon activation by ER stress, Ire1 oligomerizes resulting in trans-autophosphorylation and activation of its RNase domain (Kimata *et al.*, 2007; Aragon *et al.*, 2009; Korennykh *et al.*, 2009; Li *et al.*, 2010). In *Saccharomyces cerevisiae*, active Ire1 unconventionally splices an intron from the Hac1 mRNA in the cytosol (Cox and Walter, 1996; Mori *et al.*, 1996), resulting in de-repression of Hac1 translation and a frameshift in the resulting transcript leads to activation of the Hac1 protein (Mori *et al.*, 2000; Ruegsegger *et al.*, 2001). The Hac1-spliced (Hac1s) protein is an active transcription factor that induces large transcriptional changes that increase ER function and capacity (Travers *et al.*, 2000). In metazoans, a similar pathway is initiated when active Ire1 splices an intron from Xbp1, the Hac1 homolog (Shen *et al.*, 2001; Yoshida *et al.*, 2001; Calfon *et al.*, 2002). In both yeast and mammalian cells, Ire1 also functions in activities beyond Hac1/Xbp1 splicing,

such as regulated Ire1 dependent degradation (RIDD – see following sections) (Hollien and Weissman, 2006; Han *et al.*, 2009; Hollien *et al.*, 2009; Kimmig *et al.*, 2012; Tam *et al.*, 2014). Additionally, in mammalian cells, active Ire1 interacts with the C-Jun N-terminal kinase (Jnk) activating proteins Traf2, Ask1, and Aip-1 (Urano *et al.*, 2000; Nishitoh *et al.*, 2002; Luo *et al.*, 2008). These interactions result in activation of Jnk and its downstream signaling (Urano *et al.*, 2000).

Beyond Ire1, metazoans also rely on Perk and Atf6 for complete activation of the UPR (Moore and Hollien, 2012). Perk is an eIF2α kinase that dimerizes upon induction of ER stress, leading to trans-autophosphorylation and activation of its kinase domain (Korennykh and Walter, 2012). Once activated, Perk phosphorylates eIF2α, resulting in a decrease in general protein production through diminished regeneration of the active tRNA-met complex (Shi *et al.*, 1998; Harding *et al.*, 1999; Jackson *et al.*, 2010). Interestingly, a subset of mRNAs with upstream open reading frames (uORFs), such as Atf4, is specifically translated under these conditions. Atf4 is another transcription factor that regulates many genes involved in the secretory system (Harding *et al.*, 2000a). The third UPR transducer is Atf6 (Haze *et al.*, 1999; Wang *et al.*, 2000; Yamamoto *et al.*, 2010). Unlike Ire1 and Perk, under unstressed conditions Atf6 forms homodimers and is thought to depolymerize upon induction of ER stress (Nadanaka *et al.*, 2007). It then travels to the Golgi apparatus where site 1 and site 2 proteases cleave its cytosolic domain from the luminal domain (Ye *et al.*, 2000). The cytosolic portion of Atf6 is a third transcription factor, which works in conjunction with Xbp1 and Atf4 to increase the capacity of the ER (Figure 1.3) (Yamamoto *et al.*, 2007).



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Annu. Rev. Genet. 46:165–83

Figure 1.3 Mediators of the UPR. Summary of the three main signaling branches of the UPR. The sensors Ire1, Perk, and Atf6 detect changes in endoplasmic reticulum (ER) homeostasis and activate bZip transcription factors through unconventional mRNA splicing, translational upregulation, and intermembrane proteolysis, respectively. This transcriptional regulation, in parallel with Ire1-mediated mRNA decay and Perk-mediated translational regulation, restores and enhances ER function. Abbreviation: BiP, binding immunoglobulin protein. Reprinted with permission of Annual Review of Genetics, 46, Moore, K. A. & Hollien, J., The Unfolded Protein Response in Secretory Cell Function, 165-83, Copyright 2012.

A large amount of energy goes into enhancing ER function and capacity; however, unresolvable ER stress shifts UPR signaling towards apoptosis (Shore *et al.*, 2011). Increases in pro-apoptotic signaling through Jnk, and transcription of genes encoding pro-apoptotic proteins such as Puma, Noxa, Bim, and Chop all result from activation of various UPR mediators (Zinszner *et al.*, 1998; Li *et al.*, 2006; Puthalakath *et al.*, 2007; Upton *et al.*, 2008). These pathways are thought to converge on the intrinsic mitochondrial apoptotic pathways, and under prolonged ER stress, cleavage of apoptotic mediators such as Caspase-3 and -7 is observed (Gupta *et al.*, 2010). While pro-apoptotic signaling is induced by all three of the UPR mediators, loss of any branch of UPR signaling results in increased rate of cell death indicating that the primary function of the UPR is to alleviate the ER stress, and that apoptotic signaling is activated only when ER stress is irremediable (Harding *et al.*, 2000b; Lee *et al.*, 2003; Yamamoto *et al.*, 2010). However, the factors that signal the transition from prosurvival to pro-apoptotic processes are not well understood.

Ire1 Structure and Function

The research described in this dissertation primarily focuses on Ire1 and its ability to cleave mRNAs. The N-terminus of Ire1 resides within the ER lumen and is responsible for sensing ER stress, most likely through direct interaction with unfolded proteins (Gardner and Walter, 2011). The luminal domain is connected to its cytosolic kinase and RNase domains via a Type I transmembrane domain and a flexible linker domain. The kinase domain shares homology with the well-studied CDK2 serine/threonine class of kinases, while the

RNase domain appears to be unique (Korennykh and Walter, 2012). Ire1 resides mostly as a monomer in a complex with the Hsp70 chaperone, BiP, during unstressed conditions (Bertolotti *et al.*, 2000). BiP binds to a juxta-transmembrane region of the Ire1 luminal domain that is not required for activation of Ire1 (Kimata *et al.*, 2004). Upon accumulation of misfolded proteins, BiP is titrated off the luminal domain of Ire1, and there is increasing evidence that misfolded proteins bind directly to a groove within the Ire1 luminal domain resulting in dimerization (Credle *et al.*, 2005; Gardner and Walter, 2011). Initial Ire1 dimers form in the face-to-face configuration, which results in exchange of kinase activation loops, while the RNase domains remain distant from each other (Figure 1.4B) (Joshi *et al.*, 2015).

In current models of Ire1 activation the initial "face-to-face" dimer is considered an early intermediate stage in Ire1 activation, while the back-to-back dimer is the active conformation of Ire1 (Walter and Ron, 2011; Maly and Papa, 2014). The face-to-face dimer allows for trans-autophosphorylation of the kinase activation loops, resulting in a conformational change into a new back-to-back dimer (Figure 1.4C) (Lee *et al.*, 2008; Korennykh *et al.*, 2011a). In the back-to-back conformation the kinase activation loops face away from each other while the RNase domains form a protein interface allowing for activation (Lee *et al.*, 2008; Joshi *et al.*, 2015). Back-to-back dimers are then thought to oligomerize into clusters of upwards of 8 monomers (Aragon *et al.*, 2009; Li *et al.*, 2010). Increases in oligomerization of Ire1 result in stabilization of the Helix-loop element (HLE) of the RNase domain (Korennykh *et al.*, 2009), and *in vitro* studies have shown that increasing oligomerization correlates with increased RNase

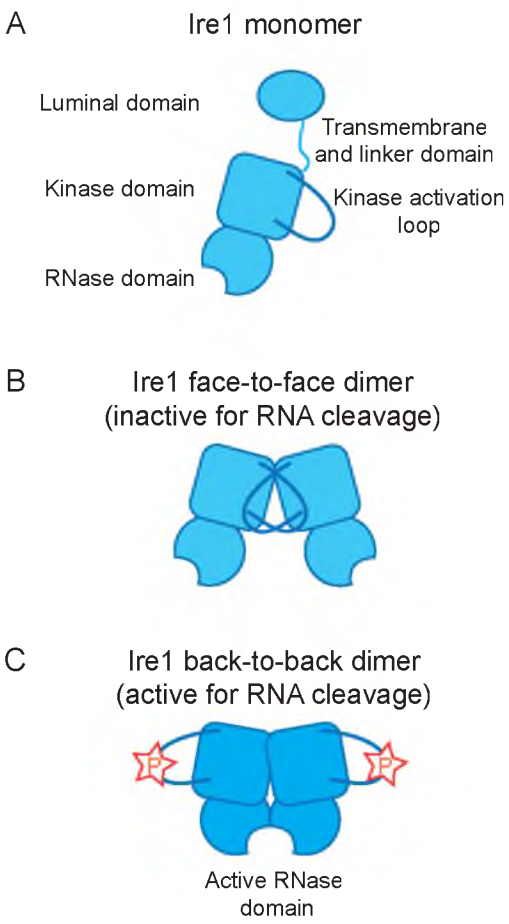

Figure 1.4 Ire1 conformations before and after activation. (A) A representation of an Ire1 monomer. (B) The kinase and RNase domains of an Ire1 dimer in the face-to-face conformation. The activation loops of the kinase domains are predicted to interact each other resulting in ATP binding and transphosphorylation. The RNase domains do not interact in this conformation; consequently RNase activity is low. (C) The kinase and RNase domains of an Ire1 dimer in the back-to-back conformation. Binding to the ATP binding pocket leads to a conformational change in the Ire1 dimer resulting in the back to back configuration in which the RNase domains interact forming a functional RNA binding pocket and high RNase activity. This conformation also allows for higher order oligomerization through interactions with the newly accessible interfaces.

activity, indicating that high order oligomers of active Ire1 are important for RNase activity (Korennykh *et al.*, 2011a). Interestingly, while over 20 phosphorylated residues have been observed on active Ire1, the functionality of the kinase domain is unnecessary for RNase activation. Rather, nucleotide binding to the kinase pocket results in the conformational changes that allow for RNase activation (Korennykh *et al.*, 2009). Incidentally many Ire1 kinase inhibiters strongly induce its RNase activity (Joshi *et al.*, 2015).

The catalytic activity of the RNase domain relies on acid-base catalysis. Proton transfer from a histidine (residue 1061 in yeast, 910 in humans), which acts as a general acid and a tyrosine (residue 1043 in yeast, 892 in humans), which acts as a general base, results in sequence-specific catalytic cleavage of mRNA (Dong *et al.*, 2001; Korennykh *et al.*, 2011b). Based on structural data, it appears that, although two active catalytic sites exist within the Ire1 back-to-back dimer, the size of the RNA binding pocket is too small to allow for cleavage of more than one RNA substrate at a time (Korennykh *et al.*, 2009). However, in order for the RNA to dock appropriately in the RNase active site stabilization derived from HLE domains of both Ire1 molecules within the dimer is required.

Hac1/Xbp1 Splicing

Cleavage of Hac1/Xbp1 is extremely sequence and structure specific. Cleavage occurs 3' of a guanosine nucleotide at the third position within the loops of a conserved dual stem loop structure within the coding region of the transcript (Sidrauski and Walter, 1997; Calfon *et al.*, 2002). Furthermore, mutations that disrupt base pairing within the stems, or either loop, also result in

cleavage inhibition. The cleavage results in a 2' 3' cyclic phosphate group, a 5' hydroxyl, and loss of an intron from the transcript (Gonzalez *et al.*, 1999). The resulting 5' and 3' ends of the cleavage site are then ligated together by tRNA ligase in yeast, and unknown and possibly redundant ligases in metazoans. In addition to sequence and structure specificity of the stem loops, the secondary structure of the mRNA surrounding the dual stem loop region is also important for Ire1 cleavage (Sidrauski *et al.*, 1996). Mutations that result in loss of secondary structure upstream and downstream of the stem loop structures also inhibit cleavage in mammalian cells (Calfon *et al.*, 2002).

Regulated Ire1 Dependent Decay (RIDD)

RIDD was originally discovered in *Drosophila* S2 cells, where a large number of ER-localized mRNAs are degraded in an Ire1-dependent, Xbp1-independent manner (Hollien and Weissman, 2006). While the biochemical mechanisms of RNA cleavage by the RIDD pathway have not been determined, similar to Xbp1 splicing, RIDD is inhibited by mutations that block access to the Ire1 RNase active site and compounds that target the nuclease activity of Ire1 (Cross *et al.*, 2012). To date, no mutations have been found that distinguish between the splicing reaction of Xbp1 and RIDD target cleavage in mammalian systems; however, evidence of differences between the two pathways do exist. For example, Xbp1 splicing happens on a much faster time scale than RIDD, with ~90% of Xbp1 transcripts being spliced within 10 minutes after the induction of ER stress, while maximum degradation through RIDD often takes several hours under the same conditions (unpublished observations). Furthermore, artificial

activation of an Ire1 mutant that binds to an ATP-analog results in only Xbp1 splicing. However, this mutant was fully competent to perform RIDD upon addition of ER stress and the ATP-analog, indicating that additional requirements beyond activation of Ire1's RNase domain must exist for RIDD (Han *et al.*, 2009; Hollien *et al.*, 2009).

Work to elucidate RIDD mechanisms and function initially focused on the cell biology of the pathway in *Drosophila* cells. Gaddam *et al.* (2013) showed that ER-localization is both necessary and sufficient to target an mRNA to RIDD in *Drosophila* cells. Because the vast majority of mRNAs that encode proteins of the transmembrane system reside at the ER, they are disproportionately sensitive to RIDD (Hollien and Weissman, 2006). These results led to the hypothesis that RIDD may result in an initial downregulation of proteins entering the ER, which would then sensitize the ER to the major increases in transcription that support ER function and capacity. Interestingly, Smt3, the SUMO homolog in flies, is also degraded through RIDD even though its mRNA is not ER-localized, indicating that degradation of specific mRNAs may also have functional consequences for the cell. Furthermore, degradation of a specific mRNA (FatP) has been shown to be essential for normal fly eye development confirming that in addition to a possible broad function of mRNA degradation, loss of specific mRNAs is also a functional consequence of RIDD in flies (Coelho *et al.*, 2013).

RIDD has also been observed both *in vivo* and *in vitro* in mammalian systems. mRNA degradation through RIDD has been observed with chemical induction of ER stress, Ire1 overexpression, and Ire1 hyperactivation resulting from depletion of Xbp1 (Han *et al.*, 2009; Hollien *et al.*, 2009; Hur *et al.*, 2012).

When Ire1 is either overexpressed or hyperactivated for extended periods of time, mammalian Ire1 cleaves numerous mRNAs that encode proteins of the secretory system, similar to patterns observed in *Drosophila* cells. However, it is clear that during acute instances of ER stress induction (i.e., chemical stressors) RIDD is much more specific than in S2 cells with only a subset of ER-localized mRNAs being targeted to the RIDD pathway (Hollien *et al.*, 2009; So *et al.*, 2012), indicating that specific mRNAs are prioritized to RIDD even though mammalian Ire1 is capable of cleaving mRNAs with less specificity. Additionally, because of the variability between mRNAs expressed in different systems and the massive remodeling of transcription that occurs during the UPR, compiling a comprehensive list of RIDD targets has remained challenging. However, of the confirmed RIDD targets in mammalian systems some similarities have been observed. Unlike S2 cells, in which unique Ire1 cleavage sequences do not seem to exist (Hollien and Weissman, 2006), mammalian RIDD targets contain Xbp1-like stem loops (Gaddam *et al.*, 2013). Mutation of the predicted cleavage site within the loop sequence of two transcripts inhibited cleavage by recombinant human Ire1 *in vitro*, indicating that cleavage by mammalian Ire1 may be more dependent on target sequence than cleavage by *Drosophila* Ire1 (Hur *et al.*, 2012; So *et al.*, 2012).

Models of RIDD Function in Mammals

In mammals, there are two nonmutually exclusive models for RIDD function. Either, nonspecific degradation of numerous RNAs, or specific degradation of a few mRNAs affects the cell's ability to respond appropriately to

stressful situations. Furthermore, localized degradation of mRNAs, possibly at contact sites between the ER and mitochondria where Ire1 has been observed (Mori *et al.*, 2013), results in reduction of proteins that are locally important. In this scenario, the local effect of RIDD may be large, but the overall changes in mRNA levels would be minimal and go largely unobserved when measuring total mRNA levels. These alternative models of RIDD function arise from the observation that Ire1 can degrade broad classes of mRNAs as observed with Ire1 overexpression or hyperactivation in mammalian cells, but under acute instances of stress, only a relatively small subset of mRNAs are degraded to a measurable degree (Han *et al.*, 2009; Hollien *et al.*, 2009; So *et al.*, 2012). Furthermore, it has not been determined whether the RIDD pathway contributes to cell survival or cell death. Evidence from a variety of techniques and systems exists to support both possibilities (Han *et al.*, 2009; Cross *et al.*, 2012; So *et al.*, 2012; Upton *et al.*, 2012). In an effort to better understand the function of the RIDD pathway we have taken a mechanistic approach to determine how mRNAs are targeted to the RIDD pathway, which will guide our future studies towards finding the biologically relevant targets of the RIDD pathway and its physiological function.

Concluding Remarks

The endoplasmic reticulum is a dynamic organelle that is remodeled to fit the needs of the cell. To ensure efficient protein processing the environment of the ER is under constant surveillance. The mediators of the UPR (Ire1, Perk, and Atf6) counteract perturbations to this system by changing the translational and

transcriptional landscape of the cell. These changes result in increases in ER size and folding ability. Modification of ER capacity is essential for responding to pathological insults as well as the general secretory needs of different cell types; however, under conditions of irremediable stress the mediators of the UPR induce apoptotic pathways to avoid the cytotoxic effects of misfolded protein release. The factors that tip the balance from cell survival to cell death signaling during ER stress are not well understood.

The RIDD pathway has been hypothesized to play a role in both pro-survival and prodeath pathways; however, its physiological function remains obscure. I have used molecular biology techniques and chemical induction of ER stress to address the question of how mRNAs are prioritized to the RIDD pathway and whether their specific degradation has physiological effects on the cell. Here I describe the requirements for RIDD targeting of both a noncanonical RIDD target in flies as well as the mechanisms employed by the RIDD pathway in mammalian cells (Chapters 2 and 3). Based on the information generated through these studies I propose a model for how RIDD signaling may function in both prosurvival and prodeath pathways depending on the type and duration of stress induced. Finally, based on our understanding of RIDD targeting I have hypothesized a role for degradation of a single mRNA, resulting in a more efficient response to ER stress (Chapter 4).

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CHAPTER 2

REGULATION OF SUMO mRNA DURING ENDOPLASMIC RETICULUM STRESS

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Regulation of Sumo mRNA during Endoplasmic Reticulum Stress

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Abstract

The unfolded protein response (UPR) is a collection of pathways that maintains the protein secretory pathway during the many physiological and pathological conditions that cause stress in the endoplasmic reticulum (ER). The UPR is mediated in part by Ire1, an ER transmembrane kinase and endoribonuclease that is activated when misfolded proteins accumulate in the ER. Ire1's nuclease initiates the cytosolic splicing of the mRNA encoding X-box binding protein (Xbp1), a potent transcription factor that then upregulates genes responsible for restoring ER function. This same nuclease is responsible for the degradation of many other mRNAs that are localized to the ER, through Regulated Ire1 Dependent Decay (RIDD). Here we show that Smt3, a homolog of small ubiquitin-like modifier (sumo), is a non-canonical RIDD target in *Drosophila* S2 cells. Unlike other RIDD targets, the sumo transcript does not stably associate with the ER membrane, but instead relies on an Xbp1-like stem loop and a second UPR mediator, Perk, for its degradation during stress.

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Introduction

The flux of proteins through the secretory pathway varies extensively among cell types and different pathological and physiological conditions. As demand for secreted proteins changes, so do the systems within the endoplasmic reticulum (ER) that are responsible for protein folding and processing. ER stress results when accumulation of unfolded proteins overcomes the folding capacity of the ER. In metazoans, this situation is sensed by three main classes of ER transmembrane proteins- Ire1, Perk, and Atf6- which together mediate the numerous changes in gene expression that define the unfolded protein response (UPR) [1,2]. This response is essential for normal development in mammals and is thought to impact several diseases, including diabetes, cancer, and neurodegenerative disorders [3].

The UPR has broad effects on transcription, translation, and mRNA decay during ER stress. Translational regulation is mediated largely by Perk, which dimerizes during ER stress and is activated through autophosphorylation [4,5]. Perk phosphorylates the translation initiation factor eIF2α, thereby inhibiting cap-dependent translation of most transcripts [6,7]. However, transcripts containing upstream open reading frames (uORFs), such as the basic-leucine zipper (b-zip) transcription factor Atf4, are selectively translated in these conditions and thus their expression increases during ER stress [8]. Ire1, a second mediator of the UPR, oligomerizes during stress, leading to activation of its cytosolic kinase and endoribonuclease domains [9,10,11]. Ire1 specifically cleaves the mRNA encoding X-box binding protein (Xbp1), directly leading to the cytosolic splicing and translation of this b-zip transcription factor [12,13]. Along with Atf4 and Atf6 (a third b-zip transcription factor activated by proteolysis during ER stress [14]), Xbp1 transcriptionally upregulates many genes encoding ER-specific protein folding chaperones and other proteins that function in the secretory pathway [15,16]. Ire1 is also necessary for cleavage of many other mRNAs, initiating their degradation through Regulated Ire1 Dependent Decay (RIDD) [17,18,19].

Although much is known about the mechanism of Xbp1 splicing, the features of mRNAs that identify them as RIDD targets have been more elusive. In *Drosophila melanogaster* cells, localization to the ER membrane appears to be the major factor in targeting mRNAs to this pathway; ER-targeting signals are both necessary and sufficient for degradation by RIDD [17,20], and there is a strong correlation between the extent of membrane association of a given mRNA and its degradation by RIDD during ER stress [20]. Conversely, cleavage site specificity does not appear to be important for RIDD targeting in *Drosophila* [20]. Based on gene ontology classifications, RIDD targets in mammals and *S. pombe* are enriched for mRNAs encoding secretory proteins, and therefore are presumed to be localized to the ER [18,19,21]. However, RNA localization does not appear to fully account for the specificity of RIDD in these organisms, suggesting that there are other targeting requirements. These requirements may include specific sequences such as the stem loop structures that define the cleavage sites in Xbp1 and are also enriched in mammalian RIDD targets [18,19,22].

Interestingly smt3, the *D. melanogaster* homolog of sumo, was identified in microarray experiments as a potential RIDD target [17], despite lacking any recognizable sequence elements that would target it to the ER. This observation led us to hypothesize that the sumo transcript may rely on different mechanisms for degradation compared to the majority of RIDD targets in flies.

Here we demonstrate that the mRNA encoding sumo is a non-canonical RIDD target and depends on both an Xbp1-like stem loop structure and Perk for its degradation during ER stress.

Results

The mRNA encoding sumo is a non-canonical RIDD target

We previously observed by microarray that the relative amount of the sumo (smt3, CG4494) transcript decreases during ER stress in *D. melanogaster* S2 cells, in an Ire1-dependent but Xbp1-independent manner [17]. We confirmed this result here by quantitative real-time PCR (qPCR) (Figure 1A-B). Depletion of either Ire1 or Xbp1 by RNAi inhibited the upregulation of BiP, a major ER chaperone, during ER stress (Figure 1A). However, depletion of Ire1 but not Xbp1 blocked the downregulation of sumo mRNA (Figure 1B). To test whether this decrease was the result of mRNA decay, we treated S2 cells with actinomycin D (1 μg/mL) to block transcription and collected samples over time in the presence and absence of dithiothreitol (DTT, 2 mM), a reducing agent that strongly induces ER stress. Tunicamycin and thapsigargin, two other strong inducers of ER stress in mammalian cells, do not efficiently activate Ire1 in S2 cells [17], thus DTT was used to activate ER stress pathways in the following experiments. Sumo mRNA levels were stable in actinomycin-treated cells over six hours, but significantly decreased over time during ER stress (Figure 1C). Therefore, sumo is a RIDD target.

While ER localization appears to be necessary and sufficient to target mRNAs to RIDD in S2 cells [17,20], sumo contains neither a signal sequence nor a transmembrane domain, and thus its mRNA cannot localize to the ER by conventional mechanisms. To determine experimentally whether this mRNA is localized to the ER through an alternative pathway, we used a previously-described detergent fractionation method [20,23] to separate membrane-bound vs. cytosolic mRNAs from S2 cells. As predicted from its sequence and the known cytosolic/nuclear functions of the sumo protein, sumo mRNA was highly enriched in the cytosolic fraction, along with the mRNA encoding actin (Figure 1D). Its fractionation behavior did not change with ER stress (Figure 1D), although as we have previously found, the membrane-associated mRNA sparc became more digitonin-extractable during ER stress, perhaps due to the concurrent attenuation of translation [20]. Interestingly, Xbp1 mRNA, which is cleaved by Ire1 during stress, also did not strongly fractionate with the membrane (Figure 1D), suggesting that strong, stable association with the ER is not absolutely required for cleavage by Ire1.

To further test a possible role for ER localization in the degradation of sumo mRNA, we treated S2 cells with puromycin (35 μM), a translation elongation inhibitor that releases mRNAs from ribosomes and disrupts the ER localization of mRNAs that rely on translation-dependent mechanisms of membrane targeting. Degradation of the sumo transcript during ER stress was not significantly affected by puromycin treatment (Figure 1E). In contrast, other RIDD targets (sparc and Tsp42Ee) were no longer degraded in the presence of puromycin, most likely because the mRNAs were no longer associated with the ER. These results suggest that ribosome-dependent membrane localization is not necessary for RIDD targeting of sumo mRNA.

An Xbp1-like stem loop is necessary and sufficient for targeting sumo mRNA to RIDD

To examine the *cis* elements in the sumo transcript important for its degradation during ER stress, we used reporter plasmids expressing the coding sequence of sumo under the control of the copper-inducible metallothionein promoter. After inducing expression of reporter mRNAs in S2 cells with $CuSO_4$, we removed the transcriptional inducer and monitored mRNA degradation in the presence and absence of ER stress. Although regulation of localization, translation, and degradation of mRNAs often relies on sequence elements within the 3′UTR, we found that replacing the sumo 3′UTR with that of sparc (an ER-localized RIDD target) or Gapdh1 (a cytosolic mRNA unaffected by ER stress) did not affect its targeting to RIDD (Figure 2A).

Further sequence analysis, however, revealed that the *D. melanogaster* sumo transcript contains a predicted stem loop near the end of its coding sequence that bears a striking similarity to the Xbp1 stem loop sequences that are cleaved by Ire1 (Figure 2B). Deletion of the 27 nucleotides surrounding this structure abolished ER stress-dependent degradation of the sumo mRNA reporter (Figure 2C). To probe this sequence more specifically, we made point mutants within the loop and the stem. Mutation of any of the 4 conserved bases within the 7-member loop [24] blocked ER stress-dependent degradation of sumo mRNA, whereas mutation of a non-conserved base in the loop had no effect (Figure 2C). Likewise, mutation of 3 nucleotides within the predicted stem structure also blocked degradation (Figure 2C).

To determine whether the Xbp1-like stem loop within the sumo transcript is sufficient for targeting an mRNA to RIDD, we used a reporter plasmid encoding GFP. The GFP mRNA alone is not a RIDD target ([20] and Figure 2D, E). However, addition of the 27 nucleotides surrounding the sumo stem loop to the 3′ end of the GFP coding sequence led to an ER stress-dependent increase in the degradation of GFP mRNA (Figure 2D). We then tested whether this degradation was Ire1 dependent by depleting Ire1 through RNAi. Degradation of the GFP mRNA alone was unaffected by Ire1 depletion, whereas the enhanced degradation of the GFP-stem loop mRNA seen during ER stress was inhibited by Ire1 depletion (Figure 2E). Thus, degradation of the GFP-stem loop mRNA occurs through RIDD.

Sumo is not a strong RIDD target in mammalian cells

To determine whether regulation of sumo mRNA by RIDD is conserved, we searched for Xbp1-like stem loops in sumo transcripts of other organisms, using the criteria that an Ire1 site must contain a stem loop with at least 5 basepairs in the stem and exactly 7 nucleotides in the loop, and must contain the four conserved loop nucleotides depicted in Figure 2B. The Ire1 site was not widely conserved; even within *Drosophila*, we found Ire1 sites in the sumo transcripts of only 2 of the 11 species we examined, namely *D. sechellia* and *D. simulans*, the closest relatives to *D. melanogaster* (Figure 3A). We did not uncover any predicted Ire1 sites in the sumo transcripts for humans, *X. laevis*, or *C. elegans*.

Despite this lack of general conservation, we did find an Ire1 site in a mouse sumo transcript. Mice possess three sumo genes, in contrast to *D. melanogaster*, which has only one. While neither sumo1 nor sumo3 contains a predicted Ire1 site, sumo2 has a stem loop at exactly the same position, relative to the coding sequence, as the one in *D. melanogaster* sumo. The loop and first four basepairs of the stem are perfectly conserved between these two transcripts (Figure 3A).

To determine whether sumo2 is downregulated during ER stress in mouse cells, we treated mouse preosteoblast MC3T3-E1 cells with DTT (2 mM, 4 hrs) and measured mRNA levels for the mouse sumo homologs by qPCR (Figure 3B). These cells robustly degrade the RIDD target Blos1 in response to ER stress. Sumo2 displayed a very weak downregulation (p-value for untreated vs. DTT-treated = 0.08). Depletion of Ire1 by RNAi blocked the degradation of Blos1, and appeared to also affect sumo2 down-

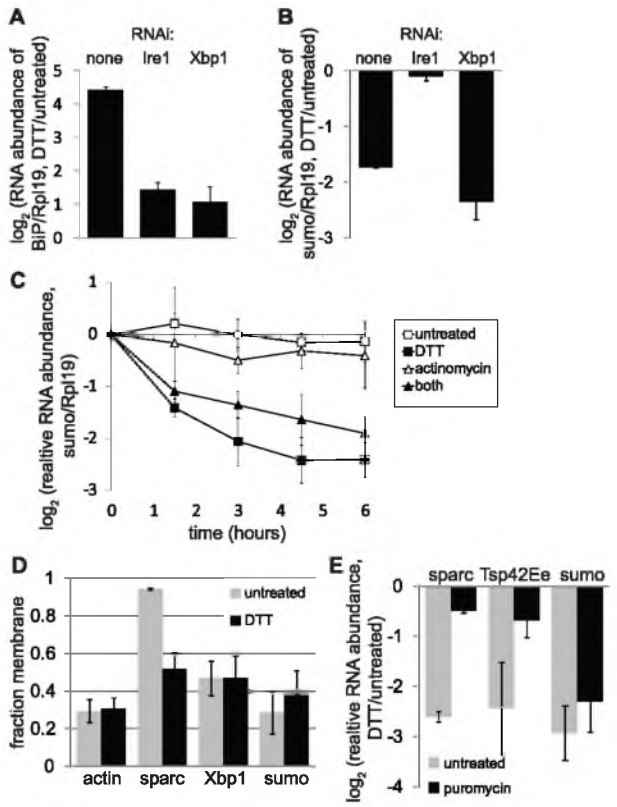

Figure 1. Sumo mRNA is a non-canonical RIDD target. For all panels, we measured relative RNA abundance by qPCR; shown are the averages and SDs of 3-4 independent experiments. Except for the fractionation in panel D, we normalized all mRNA abundance measurements to the housekeeping control Rpl19. A-B. Relative mRNA levels of BiP (panel A) and sumo (panel B) in mock-treated and Ire1- or Xbp1-depleted *Drosophila* S2 cells incubated in the absence and presence of ER stress (2 mM DTT, 4.5 hours). Xbp1 transcript levels in the Xbp1 RNAi-treated cells were 13.5% +/− 0.9% of the levels in control cells, as measured by qPCR. C. Timecourse of sumo mRNA levels in S2 cells treated with or without actinomycin D (1 μg/mL) to block transcription and DTT (2 mM) to induce ER stress. D. Fraction membrane (membrane/total) for mRNAs from S2 cells treated with and without DTT (2 mM, 20 min). We separated cytosolic and membrane RNAs using detergent extraction (see Materials and Methods). E. Relative mRNA levels in cells treated with or without 35 μM puromycin (added 10 min prior to stress) and DTT (2 mM, 4 hrs).
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regulation; however the overall effect was weak and did not pass the standard p-value cutoff for statistical significance, using a paired t-test (p-value for dtt/untreated, control vs Ire1 RNAi = 0.2).

To account for potential variation in Ire1 site preferences and test the possibility of sumo regulation in human cells, we repeated the above experiments in HEK293 cells (Figure 3C). Treatment with DTT (2 mM, 4 hrs) resulted in a small but significant decrease in sumo2 mRNA levels (p-value = 0.02). This effect was not Ire1-dependent, consistent with the lack of predicted Ire1 sites in human sumo transcripts. Levels of sumo1 and 3 remained unchanged in both MC3T3-E1 and HEK293 cell types. These results suggest that while sumo is downregulated in mammals during ER stress, the effect is small and the mechanisms regulating sumo levels vary between organisms.

RIDD of the sumo transcript is dependent on Perk

During ER stress, Perk activation and phosphorylation of eIF2α result in an attenuation of translation, which can affect mRNA stability [25,26]. To test whether Perk is important for degradation of sumo mRNA, we depleted Perk by RNAi and measured the relative abundance of endogenous sumo mRNA in the presence and absence of ER stress. Strikingly, degradation of the sumo transcript during ER stress was completely abolished in the absence of Perk (Figure 4C). Sumo mRNA levels in the absence of stress were not affected by Perk depletion (levels in Perk RNAi/

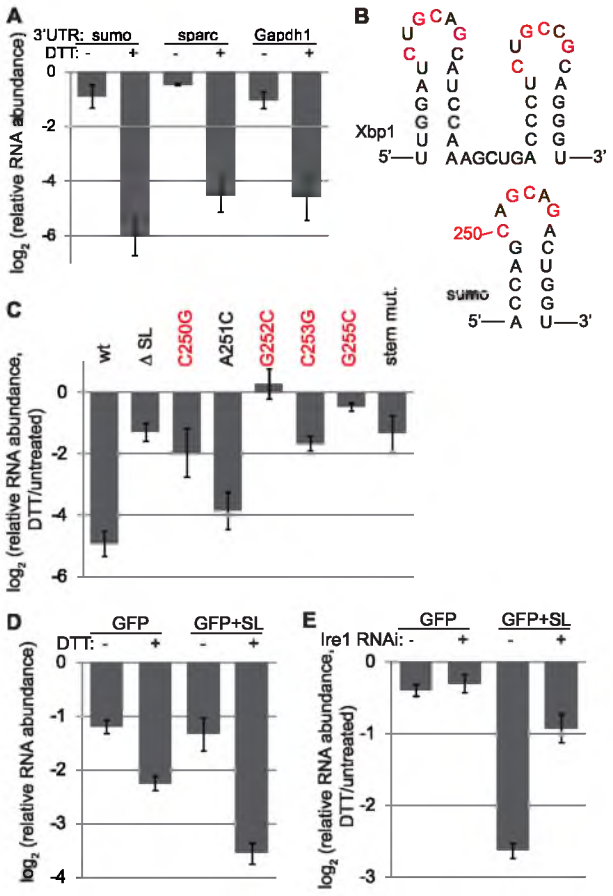

Figure 2. A stem loop sequence in the sumo mRNA is important for RIDD targeting. For panels A, C-E: plasmids expressing reporter mRNAs under the control of a copper-inducible promoter were stably transfected into S2 cells. After inducing expression, we removed the copper to stop transcription of reporter mRNAs, incubated cells in the presence and absence of ER stress (2 mM DTT, 5 hrs), and collected RNA samples. Relative RNA abundance was measured by qPCR and normalized to Rpl19. Shown are the averages and SDs of 3 (panels A, C) or 2 (panels D-E) independent experiments. A. Reporters expressing the coding sequence of sumo followed by various 3'UTRs. We normalized RNA levels to a control sample collected immediately before washing out the copper; thus RNA measurements reflect the amount of degradation after 5 hrs without copper. B. RNA sequences of sumo and Xbp1 from *D. melanogaster*, surrounding the stem loop structures discussed here. Highlighted in red are the loop nucleotides conserved in Xbp1 across species. Numbering in the sumo mRNA is relative to the translation start site. C. Reporters containing the sumo coding sequence and 3' UTR, with various mutations. ΔSL = deletion of nucleotides 244–270 in the coding sequence of sumo; stem mut. = C257G/G259C/G260C. D. Reporters expressing GFP with the Gapdh1 3'UTR, with and without the stemloop sequence of sumo (nt 244–270). E. Degradation of reporters from D in untreated cells and those depleted of Ire1.
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control cells = 0.84, p = 0.5). Furthermore, Xbp1 splicing (Figure 4B) and degradation of RIDD targets CG3984, Hydr2, and sparc (Figure 4C) were largely unaffected, although degradation of CG6650, another RIDD target, was partially inhibited. Western blot analysis of the phosphorylation of eIF2α confirmed the efficient knockdown of Perk (Figure 4A), whose mRNA levels were reduced to 39% +/− 7% compared to controls, as measured by qPCR. These data suggest that Ire1-dependent degradation of sumo mRNA is particularly sensitive to Perk activity.

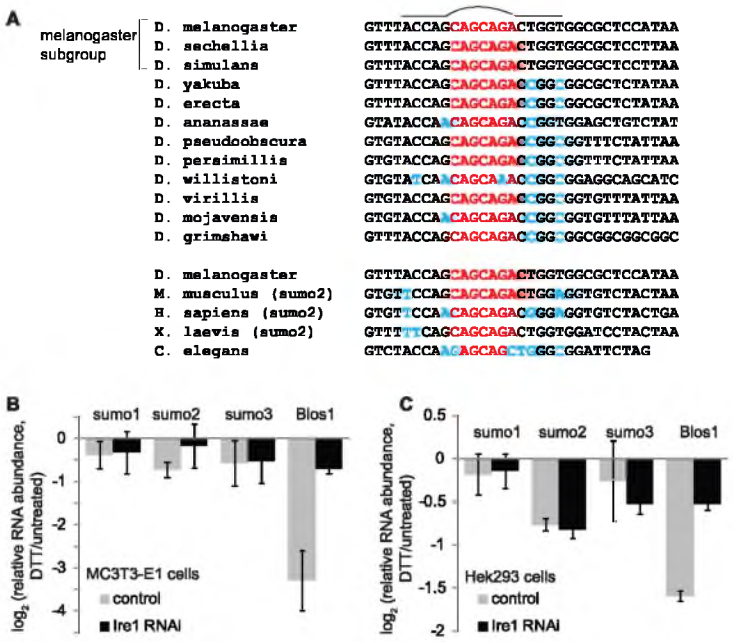

Figure 3. Sumo mRNA is not strongly affected by ER stress in mammalian cells. A. Conservation of the Ire1 site and surrounding region in sumo transcripts across species. The stem loop is indicated above the sequences and the region aligning with the loop from *D. melanogaster* sumo is shown in red. Deviations from the *D. melanogaster* sequence in the stem loop region are shown in blue. While most species have a conserved loop sequence, perfect basepairing in the stem is present only in the fly and mouse sequences. B-C. We either mock-treated (control) or used siRNA to deplete Ire1α from MC3T3-E1 mouse osteoblastic fibroblasts (panel B) or Hek293 human kidney cells (panel C). We then compared RNA levels in the presence and absence of DTT (2 mM, 4.5 hrs), by qPCR. Blos1, a RIDD target in mouse and humans, is shown as a control. Except for Blos1, the differences in mRNA levels between control and Ire1 siRNA-treated cells were not statistically significant. Shown are the averages and SDs for 2–3 independent experiments.
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Discussion

ER stress occurs in many physiological and pathological conditions, and the response to accumulation of misfolded proteins can determine cell fate. While much is known about the initiation and downstream effects of transcriptional regulation of mRNAs during the UPR, the features that target mRNAs to the RIDD pathway are less well understood. We previously found that in *Drosophila* S2 cells, ER localization is both necessary and sufficient for targeting mRNAs to RIDD [17,20], while recognizable Ire1 cleavage sites are not predictors of RIDD targeting [20]. Furthermore, previous mutagenesis experiments found a distinct lack of specific sequence elements affecting degradation, other than ER-targeting signal sequences [17]. Here we demonstrate that the mRNA encoding sumo is an exception to these rules. Although the sumo transcript is degraded by RIDD, it is not stably associated with membranes. Degradation strongly depends on a specific *cis* element in the sumo coding sequence, comprised of a stem loop structure very similar to the conserved Ire1 recognition sites in Xbp1, and mutagenesis of the conserved bases within this stem loop inhibits degradation of the transcript. This parallels mutagenesis experiments showing that these same conserved bases within the Hac1 (the Xbp1 homolog in yeast) and Xbp1 stem loops are important for cleavage and splicing [27,28,29].

This distinct targeting mechanism suggests that downregulation of sumo serves a different function during ER stress, compared to the degradation of other RIDD targets, which may relieve stress by reducing the protein folding load on the ER. The sumo protein covalently modifies many target proteins, often affecting protein localization and activity [30]. Interestingly, the spliced version of mouse Xbp1 can be SUMOylated, leading to a decrease in transcriptional activation of target genes [31,32]. Thus during ER stress, degradation of sumo mRNA may enhance UPR signaling. However, the stem loop structure shown here to be critical for RIDD of sumo does not appear to be widely conserved beyond *D. melanogaster*, and sumo mRNA is only very weakly down-regulated in the mammalian cells we have tested.

Sumo regulation is highly sensitive to Perk, as its degradation is completely abolished when Perk is depleted. This is in contrast to canonical *Drosophila* RIDD targets, which are only mildly sensitive to Perk depletion (Figure 4C and [17]). The mechanisms that mediate Perk's effect on sumo targeting to RIDD are unclear. Interactions between translational regulation and the RIDD

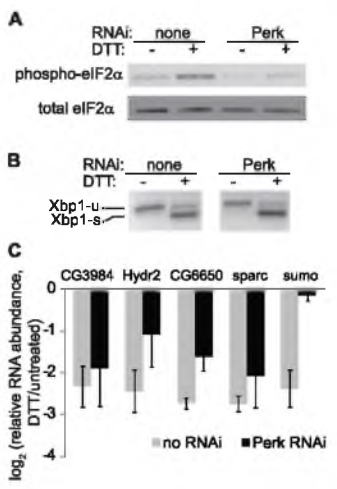

Figure 4. RIDD of sumo is dependent on Perk. We used RNAi to deplete S2 cells of Perk. A. Western blot showing the levels of phosphorylated and total eIF2α. B. Agarose gel showing the relative levels of unspliced and spliced Xbp1, amplified by reverse-transcription-PCR using primers surrounding the splice site. C. Relative mRNA levels in ER stress treated vs untreated cells, determined by qPCR. Panels A-B show representative data, panel C is the average and SD of 3 independent experiments.
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pathway are not unprecedented, as protection from translational attenuation is one way by which mRNAs at the ER membrane can escape degradation by RIDD [20]. It is possible that translation of the sumo transcript is especially attenuated when Perk is activated, or that its degradation is especially reliant on this attenuation, perhaps facilitating the formation of the sumo stem loop structure or allowing Ire1 greater access to the mRNA through ribosome depletion. It is also possible that translation attenuation is generally required for RIDD, but sumo is uniquely unaffected by Perk-independent mechanisms of attenuation that occur during ER stress in S2 cells [33]. Beyond attenuation of general translation, Perk-dependent eIF2α phosphorylation also enhances translation of certain mRNAs such as Atf4 and Gadd34 [8,34]. It is unlikely that such a protein is mediating Perk's effect on sumo degradation, as sumo mRNA is still degraded during ER stress when translation is inhibited (Figure 1E). Because we have not specifically examined transcription of sumo in Perk-depleted cells, it is also possible that Perk knockdown indirectly affects sumo transcription by an unknown mechanism.

Overall, we propose that while ER localization is a key factor in targeting most mRNAs for RIDD in *D. melanogaster* cells, stable membrane association can be overcome by the presence of a specific Ire1 recognition site coupled with translational attenuation via Perk. Although this appears to be an exception to the general RIDD targeting rules in flies, this mechanism may be more prevalent in other organisms. RIDD targets in all systems studied so far are enriched for mRNAs that are predicted to localize to the ER [17,18,19,35], but mammalian RIDD targets are also enriched for mRNAs containing Xbp1-like stem loops [18,19,22]. Interestingly, while preferred cleavage sites of several RIDD targets have been determined in both *D. melanogaster* and *S. pombe*, mutagenesis of these sites results in cleavage at alternative sites allowing for degradation to still occur [17,21]. In contrast, mutagenesis of residues important for cleavage of at least two mammalian RIDD targets inhibits their degradation *in vitro* [22,36]. These correlations suggest that while sumo regulation by RIDD does not appear to be widely conserved, the targeting mechanism exemplified by sumo may be more generally applicable to RIDD in other organisms, including mammals.

Materials and Methods

Cell culture, ER stress induction, and RNAi

We cultured *Drosophila* S2 cells (Invitrogen) at room temperature in Schneider's media (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics. Unless otherwise indicated, we induced ER stress for 5 hours with 2 mM DTT.

For RNAi experiments, we amplified regions of the Ire1 (CG4583), Perk, and Xbp1 coding sequences (CDS) from S2 cell cDNA using primers containing T7 RNA polymerase sites on the 5′ ends. We then synthesized dsRNA from these templates using the Megascript T7 kit (Ambion). We incubated S2 cells with dsRNA in serum free media for 45 minutes, replaced the serum, and allowed cells to recover for 4 days. We then repeated the dsRNA treatment and subjected cells to ER stress one day following the second dsRNA treatment.

We cultured MC3T3-E1 (ATCC) and Hek293 (from A.V. Maricq lab) cells following ATCC guidelines in MEMα and DMEM media (Invitrogen), respectively, supplemented with 10% fetal bovine serum and antibiotics. For Ire1 knockdown experiments we used organism-specific Ire1 siRNA (Qiagen) and followed Invitrogen RNAimax guidelines for transfection of siRNA. We subjected cells to ER stress 48–72 hours after transfection, when cells were approximately 80% confluent, and collected RNA after 4 hours.

Quantitative real-time PCR

For all RNA analyses, we isolated total RNA using Trizol reagent (Invitrogen), and synthesized cDNA using 2 μg of total RNA as a template, T18 as a primer, and M-MuLV reverse transcriptase (NEB). We measured relative mRNA abundance by real time quantitative PCR using a Mastercycler ep realplex (Eppendorf) with SYBR Green as the fluorescent dye. We measured each sample in triplicate and normalized to the ribosomal protein Rpl19 mRNA. To control for contaminating plasmid or genomic DNA we also measured samples to which no reverse transcriptase was added. The primers used for qPCR are given in Table 1.

Digitonin fractionation

To separate membrane and cytosolic mRNAs we used a method developed by Stephens and Nicchitta [23] and modified in our previous studies [20]. Briefly, we incubated S2 cells with or without DTT (2 mM, 20 minutes), added cycloheximide (35 μM) for 10 min, and collected cells by centrifugation. We then resuspended cells in cytosol buffer (150 mM KOAc, 20 mM Hepes pH 7.5, 2.5 mM $Mg(OAc)_2$, 200 U/mL RNaseOUT, 35 μM cycloheximide) containing 1 mg/mL digitonin (15 min on ice). We then centrifuged the lysates (800 xg, 5 min at 4 C) and collected the supernatant as the cytosolic fraction. We resuspended the pellet in cytosol buffer with 1% Triton X-100 (15 min on ice), centrifuged as above and collected the supernatant as the membrane-bound fraction. We measured the abundance of specific RNAs in each fraction by qPCR and calculated the

Table 1. Primers used for qPCR.

Gene Name	Primer1	Primer 2
Dm sumo (smt3)	TTTGTTATTTACGCACACAGACG	GTCTGACGAAAAGAAGGGAGG
Dm Ribosomal Protein L19 (Rpl19)	AGGTCGGACTGCTTAGTGACC	CGCAAGCTTATCAAGGATGG
Dm Act5C (actin)	ATGTGTGACGAAGAAGTTGCT	GAAGCACTTGCGGTGCACAAT
Dm sparc	AAAATGGGCTGTGTCCTAACC	TGCAGCACAATCTACTCAATCC
Dm Xbp1	GGCCATCAACGAGTCACTGCT	TGTGTCCACCTGTTGTATACC
Dm Tsp42Ee	AACAACGTGCGTAACTACAAGC	TTCCAAATTTAAATCTTTCCCG
Dm CG3984	CTACTGTTGTTCCTGGTACCCC	CTGGTTGCTCAGTAACACTTGG
Dm Hydr2	CGCATACACGACTATTTAACGC	TTTGGTTTCTCTTTGATTTCCG
Dm CG6650	ACAATGGGACAGGCAAAGAC	GGTGACATTCGTTTCCGAGT
Dm sumo reporters	CAGTGCAACTAAAGGGGGGATC	TTTGTTATTTACGCACACAGACG, or TCCGTCGCGGCCGCTTATGGAGCGCCACCAGTCTGCT
GFP reporters	CCTGAAGTTCATCTGCACCA	TGCTCAGGTAGTGGTTGTCG
Mm Rpl19	CTGATCAAGGATGGGCTGAT	GCCGCTATGTACAGACACGA
Mm and Hs sumo1	GGAGGCAAAACCTTCAACTG	CCCCGTTTGTTCCTGATAAA
Mm sumo2	GGGAGCCTGCTACTTTACTCC	TCCATCTCATGTCAACCAGAA
Mm sumo3	GATGGCTCGGTGGTACAGTT	TGTCCTCATCCTCCATCTCC
Mm and Hs Blos1	CAAGGAGCTGCAGGAGAAGA	GCCTGGTTGAAGTTCTCCAC
Hs Rpl19	ATGTATCACAGCCTGTACCTG	TTCTTGGTCTCTTCCTCCTTG
Hs sumo2	AGCTGAGGAGACTCCGGCGCTCGC	AGTAGACACCTCCCGTCTGC
Hs sumo3	AGAATGACCACATCAACC	AGTAGACACCTCCCGTCTGC

Dm = Drosophila melanogaster. Mm = Mus musculus. Hs = Homo sapiens.
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fraction membrane as the abundance of a particular mRNA in the membrane-bound fraction divided by the sum of that mRNA's abundance in the cytosolic and membrane-bound fractions.

Plasmids and reporter RNA analyses

For sumo reporters, we amplified the sumo (smt3, CG4494) CDS from S2 cell cDNA and subcloned into an expression vector containing the copper-inducible *D. melanogaster* metallothionein promoter described previously [17]. To examine the effects of the 3′UTR, we separately amplified the 3′UTRs of sumo, sparc (CG6378), and Gapdh1 (CG12055) from S2 cDNA and subcloned into the sumo expression vector just downstream of the CDS. We introduced mutations into the sumo vector containing the sumo 3′UTR for Figure 2C using PCR-based mutagenesis. For GFP reporters, we used a previously-described EGFP reporter in the copper-inducible expression vector [20], and replaced the vector SV40 3′UTR with that from Gapdh1. To introduce the sumo SL, we added the 30 nucleotide sequence from the 3′ end of the sumo CDS (including the stop codon) in-frame to the 3′ end of the GFP CDS.

We generated stable, polyclonal cell lines by cotransfecting our expression plasmids (1.8 μg) with a puromycin resistance plasmid (0.2 μg) using Cellfectin II (Invitrogen) and selecting for resistant cells. To monitor decay of mRNAs expressed from reporter constructs, we treated cells with $CuSO_4$ (200 μM overnight) to induce expression, collected "time 0" RNA samples, then washed cells to remove the $CuSO_4$. We have previously shown this procedure to be effective in blocking transcription of the reporter mRNA, such that subsequent measurements reflect decay rates [20]. We then either left cells untreated or added DTT (2 mM) and collected RNA samples after 5 hours. RNA abundance measurements were normalized to the level of RNA in the $CuSO_4$-treated cells.

Western blot analysis

We washed cells in PBS before lysing in 1x RIPA buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 1% NP-40, 1% Na-deoxycholate, and 0.1% SDS) with protease inhibitors (Thermo scientific). We resolved protein on NuPage Bis-Tris 4–12% gels (Invitrogen), transferred them to nitrocellulose membranes and probed for total eIF2α (abcam, 1:500) or Ser51-P eIF2α (abcam 26197, 1:1000) followed by a secondary IRDye 800CW antibody (Licor 926-32210, 1:10000). We visualized immunoblots using a Licor Odyssey imager.

Xbp1 splicing assay

Using S2 cDNA as a template we assayed Xbp1 splicing through PCR analysis of a fragment of the Xbp1 transcript encompassing the 23 nucleotide splice site. We resolved the spliced and unspliced products using a 2% agarose gel. Primers for this assay are shown in Table 1.

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Author Contributions

Conceived and designed the experiments: KAM JJP DG JC JH. Performed the experiments: KAM JJP DG JC JH. Analyzed the data: KAM JJP DG JC JH. Wrote the paper: KAM JH.

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CHAPTER 3

IRE1-MEDIATED DECAY IN MAMMALIAN CELLS RELIES ON mRNA SEQUENCE, STRUCTURE, AND TRANSLATIONAL STATUS

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MBoC | ARTICLE

Ire1-mediated decay in mammalian cells relies on mRNA sequence, structure, and translational status

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ABSTRACT Endoplasmic reticulum (ER) stress occurs when misfolded proteins overwhelm the capacity of the ER, resulting in activation of the unfolded protein response (UPR). Ire1, an ER transmembrane nuclease and conserved transducer of the UPR, cleaves the mRNA encoding the transcription factor Xbp1 at a dual stem-loop (SL) structure, leading to Xbp1 splicing and activation. Ire1 also cleaves other mRNAs localized to the ER membrane through regulated Ire1-dependent decay (RIDD). We find that during acute ER stress in mammalian cells, Xbp1-like SLs within the target mRNAs are necessary for RIDD. Furthermore, depletion of Perk, a UPR transducer that attenuates translation during ER stress, inhibits RIDD in a substrate-specific manner. Artificially blocking translation of the SL region of target mRNAs fully restores RIDD in cells depleted of Perk, suggesting that ribosomes disrupt SL formation and/or Ire1 binding. This coordination between Perk and Ire1 may serve to spatially and temporally regulate RIDD.

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INTRODUCTION

The endoplasmic reticulum (ER) is the entry point for proteins targeted to the secretory pathway. Secreted proteins are translated from mRNAs localized to the cytosolic face of the ER membrane and enter the ER as nascent chains that are folded and modified before exiting the organelle. The flux of proteins through the ER varies extensively among cell types and environments. Changes in this flux can result in ER stress, an imbalance between the load of unfolded proteins entering the ER and the capacity of the organelle to fold and modify them efficiently. In metazoans, ER stress activates three ER transmembrane proteins: inositol-requiring 1 (Ire1), PKR-like endoplasmic reticulum kinase (Perk), and activating transcription factor 6 (Atf6), which coordinate a signaling network known as the unfolded protein response (UPR; Walter and Ron, 2011). Although ER stress results from a variety of pathological conditions, loss of individual UPR sensors also affects normal development and physiology in several model organisms (Moore and Hollien, 2012).

Perk directly phosphorylates eukaryotic translation initiation factor 2 α (eIF2α), which leads to the attenuation of translation initiation and limits the protein-folding load on the ER (Harding *et al.*, 1999). This phosphorylation event also leads to translational up-regulation of certain proteins, including activating transcription factor 4 (Atf4) (Harding *et al.*, 2000). Concurrently, Ire1 oligomerizes in response to ER stress, activating its cytosolic nuclease domain (Li *et al.*, 2010), and cleaves the mRNA encoding X-box binding protein 1 (Xbp1). This cleavage occurs at two specific sites in a dual stem-loop (SL) structure (Yoshida *et al.*, 2001; Calfon *et al.*, 2002). The resulting 5′ and 3′ fragments are then ligated, forming a spliced transcript encoding the active transcription factor, which, together with other UPR transcription factors, up-regulates numerous genes that increase the capacity of the secretory pathway (Travers *et al.*, 2000; Harding *et al.*, 2003).

Ire1 is also responsible for the cleavage of other ER-localized mRNAs, leading to their degradation through regulated Ire1-dependent decay (RIDD; Hollien and Weissman, 2006; Hollien *et al.*, 2009). RIDD was originally observed in *Drosophila melanogaster* S2 cells, where a large number of mRNAs associated with the ER are degraded during ER stress (Hollien and Weissman, 2006). RIDD is important for *Drosophila* eye development, confirming a

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Abbreviations used: ER, endoplasmic reticulum; Ire1, inositol-requiring enzyme 1; Perk, PKR-like endoplasmic reticulum kinase; qPCR, quantitative real-time PCR; RIDD, regulated Ire1-dependent decay; SL, stem loop; UPR, unfolded protein response; Xbp1, X-box binding protein 1.

Supplemental Material can be found at:
http://www.molbiolcell.org/content/suppl/2015/06/21/mbc.E15-02-0074v1.DC1.html

physiological role for this pathway in vivo (Coelho *et al.*, 2013). In S2 cells, ER localization of an mRNA is both necessary and sufficient for its degradation by RIDD (Gaddam *et al.*, 2013). However, exceptions to this rule exist. For example, the *Drosophila* transcript encoding small ubiquitin-modifier (Sumo) is targeted to RIDD despite localizing to the cytosol. This mRNA requires an Xbp1-like SL in its coding region to be degraded by Ire1 (Moore *et al.*, 2013). In addition, for unknown reasons, RIDD of Sumo requires Perk (Moore *et al.*, 2013), even though Perk depletion does not appear to generally affect RIDD in S2 cells (Hollien and Weissman, 2006).

RIDD also occurs in mammalian cells (Han *et al.*, 2009; Hollien *et al.*, 2009). Activation of Ire1 through overexpression in cultured cells or tissue-specific Xbp1 mutations in mice, which result in hyperactivation of Ire1, induces broad cleavage of ER- localized mRNAs (Han *et al.*, 2009; So *et al.*, 2012). However, during chemical induction of ER stress in both mammalian cell culture and mice, the magnitude of degradation and number of mRNAs targeted to the pathway are more limited than in S2 cells (Hollien *et al.*, 2009; So *et al.*, 2012). This restriction of RIDD substrates suggests a dependence on additional factors or sequence elements beyond mRNA localization to the ER. One likely requirement is the presence an Xbp1-like SL within the target mRNA sequence. These SLs are more prevalent in mammalian RIDD targets than in those of *D. melanogaster* (Gaddam *et al.*, 2013). Furthermore, mutation of a conserved guanine (G) within the loop blocks mRNA cleavage by human Ire1 in vitro (Hur *et al.*, 2012) and also affects the regulation of at least one RIDD target in human cells (Bright *et al.*, 2015).

In this study, we investigate the mechanism and substrate selectivity of RIDD during acute, chemically induced ER stress in mammalian cells and describe an unexpected role for Perk in the RIDD pathway.

RESULTS

RIDD targeting in different cell types

Previous work in mammalian cells suggested that the extent of degradation of RIDD targets in the absence of Ire1 overexpression is fairly small, on the order of twofold (Hollien *et al.*, 2009). We first asked whether this result was cell-line dependent. We treated several different mammalian cell lines with chemical inducers of ER stress: dithiothreitol (DTT), which blocks disulfide bonding; thapsigargin (Tg), which depletes ER calcium reserves; and tunicamycin (Tm), an inhibitor of N-linked glycosylation. We then used quantitative real-time PCR (qPCR) to measure the stress-dependent changes in the relative levels of mRNAs that were previously identified as RIDD targets in mouse fibroblasts (Hollien *et al.*, 2009; Figure 1, A and B). Note that because our qPCR expression data are inherently ratiometric, we use a log 2 scale throughout this article, meaning that a unit of 1.0 refers to a twofold change in expression.

Xbp1 splicing was nearly complete in all stress conditions tested (Figure 1C). However, the extent of RIDD targeting varied among individual mRNAs and among the different cell types (Figure 1, A and B). In both human cell lines tested (Hek293 and Hep G2), Blos1 was degraded during ER stress, but other mouse RIDD targets were either not degraded (Scara3) or not expressed to detectable levels (Col6a1 and Hgsnat; Figure 1B). Of note, the mouse Scara3 transcript contains an Xbp1-like SL, but the human transcript does not.

We observed the most robust RIDD in MC3T3-E1 cells, a preosteoblast cell line derived from mouse calvaria (Kodama *et al.*, 1981), and therefore used these cells for further study. Using small interfering RNA (siRNA)–mediated silencing, we verified that the down-regulation of RIDD targets was Ire1 dependent and Xbp1 independent (Figure 1, D–G).

Xbp1-like stem loops are necessary for RIDD in mammalian cells

To test the importance of Xbp1-like SLs for RIDD in a cellular context, we used a reporter-based approach. We created plasmids expressing the coding sequences (CDSs) of the mouse RIDD targets Hgsnat and Blos1 with vector-derived 5′ and 3′ untranslated regions (UTRs) and stably transfected them into MC3T3-E1 cells. After treatment of cells with or without DTT (2 mM, 4 h), we measured the relative abundance of the reporter mRNAs by qPCR, using primers that spanned the CDSs and reporter UTRs and therefore did not amplify the endogenous transcripts. As expected, the mRNAs expressed from both of these plasmids were down-regulated during ER stress (Figure 2, B and C), indicating that the CDS is sufficient for RIDD of these transcripts.

The CDSs of Blos1 and Hgsnat contain Xbp1-like SLs (Figure 2A), as defined by a seven-nucleotide (nt) loop with the four conserved residues essential for Xbp1 splicing (Calfon *et al.*, 2002) and a stem of at least four consecutive base pairs (allowing for AU, GC, and GU pairs). To test whether these sites were important for RIDD, we mutated the putative Ire1 cleavage site G to cytosine (C) and measured reporter degradation. For Blos1, this mutation, as well as mutation of a second conserved loop residue, completely ablated degradation (Figure 2C). For Hgsnat, mutation of the putative cleavage site in one of the two SLs (Hgsnat SL #1) blocked RIDD (Figure 2B), whereas the corresponding mutation in a second SL (Hgsnat SL #2) did not affect its degradation during ER stress (Figure 2B). The stem of Hgsnat SL #2 is shorter and has fewer GC pairs than Hgsnat SL #1 (Figure 2A), suggesting that the stability of the stem is important for RIDD. To test this, we made mutations that disrupted the base-pairing of the Xbp1-like SL of our Blos1 reporter. These mutations blocked RIDD targeting (Figure 2D). Restoring base-pairing within the putative stem region with complementary mutations that preserved the GC content of the SL restored RIDD. However, mutations that replaced GC pairs with AU pairs prevented RIDD (Figure 2D). Together these results indicate that both the sequence and stability of Xbp1-like SLs are important for RIDD in mouse cells, as suggested previously for human cells (Bright *et al.*, 2015).

To ensure that reporter expression levels did not influence RIDD, we measured the level of overexpression of Blos1 mRNA in our reporter cell lines. Total Blos1 mRNA abundance was measured by qPCR using primers that annealed within the CDS of the Blos1 transcript and therefore amplified both endogenous and reporter mRNAs. The overexpression of the Blos1 reporter mRNAs varied from ~4- to 32-fold above endogenous Blos1 levels, which were measured using a control cell line transfected with green fluorescent protein (GFP). However, there was no correlation between reporter expression level and degradation during ER stress. Furthermore, we created two independent cell lines that expressed WT Blos1 to different levels (4- vs. 32-fold overexpression) and observed no difference in the extent of the reporter mRNA degradation during stress (Figure 2E).

An Xbp1-like stem loop is sufficient to target GFP mRNA to RIDD

To determine whether an Xbp1-like SL is sufficient to induce degradation of a transcript not normally targeted to the RIDD pathway, we used reporters expressing either GFP or an ER-targeted GFP (ssGFP) containing the signal sequence from *Drosophila* Hsp70-3. In S2 cells, this ssGFP mRNA reporter (but not the cytosolic GFP mRNA) is degraded by RIDD (Gaddam *et al.*, 2013). Similarly, rat cells overexpressing Ire1 degrade an ER-targeted

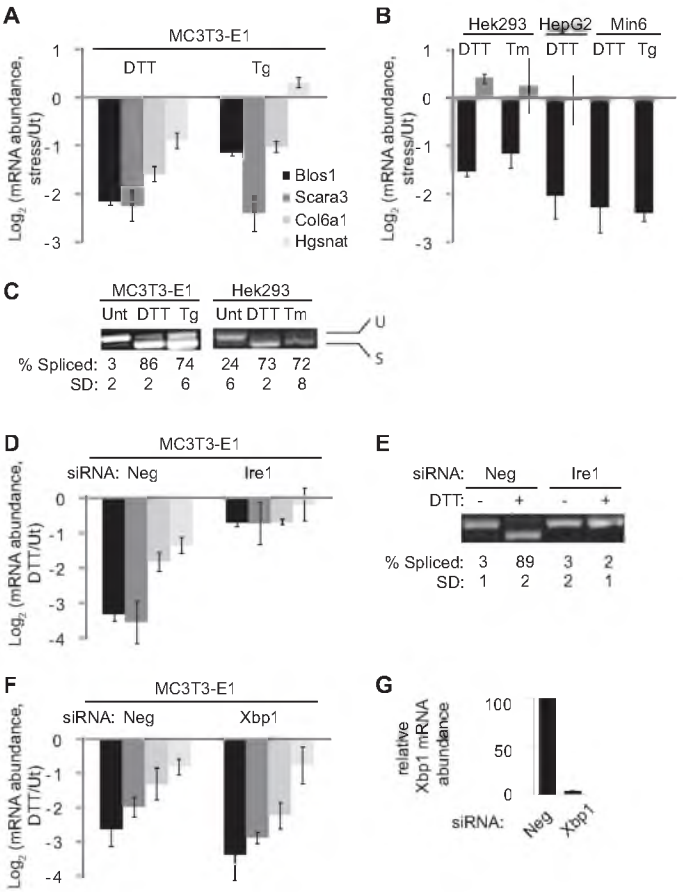

FIGURE 1: The RIDD pathway varies across mammalian cell lines. For all abundance measurements, mRNA was reverse transcribed and measured by qPCR and data were normalized to the housekeeping control mRNA Rpl19. The legend in A applies to bar graphs in A, B, D, and F. (A) Relative mRNA levels of RIDD targets in mouse MC3T3-E1 cells treated with either DTT (2 mM) or Tg (2 µM) for 4 h to induce ER stress. (B) Relative mRNA levels of Blos1 (black) and Scara3 (gray) in the indicated cell lines treated with DTT (2 mM), Tm (2.5 µg/ml), or Tg (2 µM) for 4 h. Note that Scara3 was not expressed strongly enough in Min6 cells to measure mRNA levels. (C) Samples from A and B were amplified by PCR using primers surrounding the Xbp1 splice sites. Shown are representative agarose gels with the spliced and unspliced products and averages and SDs of the percentage spliced Xbp1 for three independent experiments. (D) Relative mRNA levels of RIDD targets in MC3T3-E1 cells transfected with either Neg (negative control) or Ire1 siRNAs and then treated with or without DTT (2 mM, 4 h). (E) Xbp1 splicing in samples from D. (F, G) Relative mRNA levels of RIDD targets (F) and Xbp1 (G) in MC3T3-E1 cells transfected with Neg or Xbp1 siRNAs and then treated with or without DTT (2 mM, 4 h). Shown in all panels are the averages and SDs from two (Hek293 cells, Tm treatment) or three (all other panels) independent experiments. Ut, untreated.

GFP mRNA (Han *et al.*, 2009). However, in MC3T3-E1 cells, neither GFP nor ssGFP transcripts were down-regulated during ER stress (Figure 2F), supporting the idea that mRNA membrane association is not sufficient for RIDD in mammalian cells during acute ER stress.

To confirm that reporter mRNAs were correctly localized, we used detergent fractionation to separate membrane-associated versus cytosolic mRNAs, as described previously (Stephens *et al.*, 2008; Gaddam *et al.*, 2013). As expected, ssGFP mRNA fractionated predominately with the membrane, along with a membrane-bound control, BiP. In contrast, GFP mRNA fractionated predominantly with the cytosol, similarly to the control glyceraldehyde 3-phosphate dehydrogenase (Gapdh; Figure 2G).

Addition of the Blos1 SL to the 3′ UTR of either GFP reporter mRNA (GFP-SL_{UTR} or ssGFP-SL_{UTR}) resulted in its Ire1-dependent degradation during ER stress (Figure 2, F and H), indicating that an Xbp1-like SL is sufficient to target GFP mRNA to RIDD. Addition of the SL also resulted in a partial shift of GFP mRNA localization toward the membrane fraction (Figure 2G), suggesting that the SL alone may mediate membrane association.

Xbp1-like SLs do not predict RIDD targets generally

On the basis of these results, we hypothesized that endogenous mRNAs with Xbp1-like SLs would be RIDD targets. Previous work in mammalian cells has not led to a comprehensive list of RIDD targets, in part because transcription is highly regulated during ER stress and complicates the global analysis of mRNA degradation. Therefore we carried out a limited test of our hypothesis by blocking transcription in MC3T3-E1 cells using actinomycin D (2 µg/ml) and then measuring the relative degradation of several mRNAs in the presence and absence of DTT (1 mM, 4 h). We chose mRNAs that met the following criteria: 1) they were expressed in MC3T3-E1 cells (Nabavi *et al.*, 2012), 2) they were associated with Gene Ontology terms indicating ER, Golgi, lysosome, plasma membrane, or extracellular localization of the encoded protein, and 3) they contained strong Xbp1-like SLs with at least three GC base pairs in the stem. Surprisingly, none of the 10 mRNAs we measured was degraded more strongly during ER stress (Figure 3). These results indicate that although the presence of an Xbp1-like SL is sufficient to target GFP mRNA to RIDD, it is not generally sufficient to target endogenous mRNAs to RIDD and additional targeting features must exist.

Perk-mediated attenuation of translation is important for RIDD

Previously we determined that the noncanonical *Drosophila* RIDD target Sumo relies on both a SL and the presence of Perk to be degraded during ER stress (Moore *et al.*, 2013). To determine whether

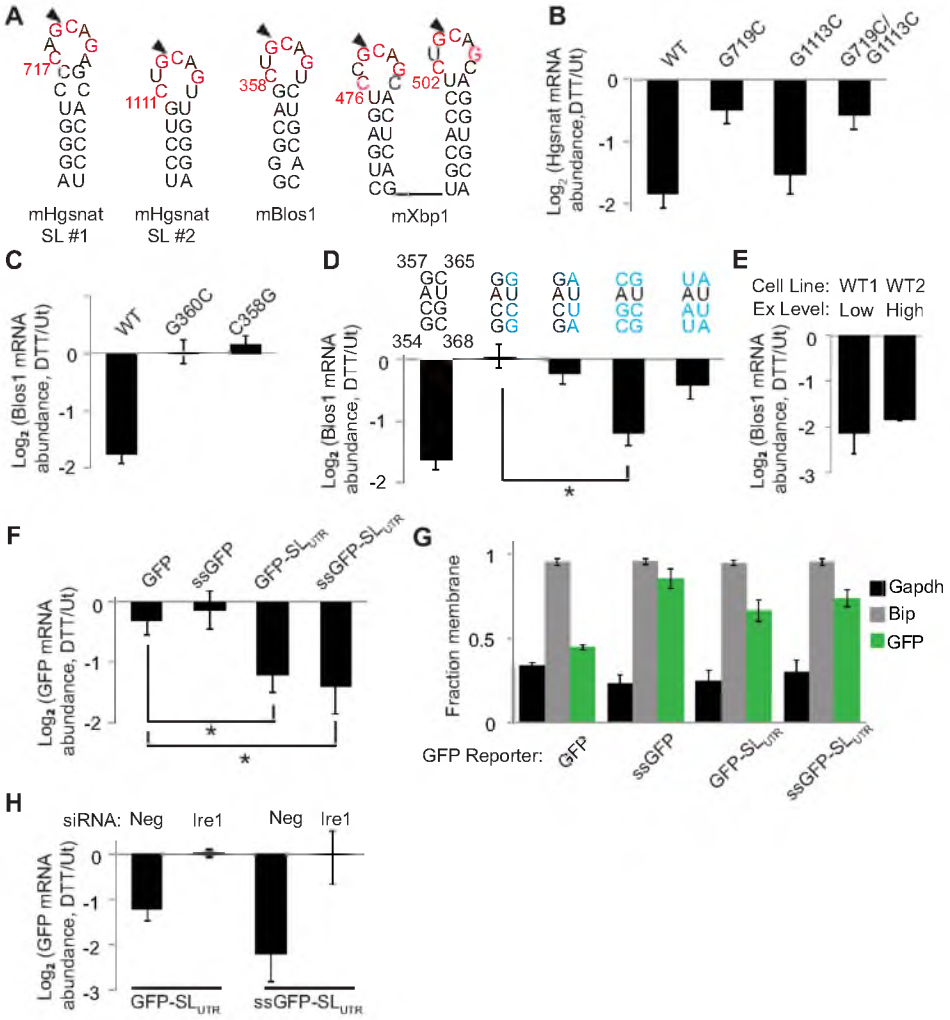

FIGURE 2: An Xbp1-like stem loop is necessary for RIDD and sufficient to induce GFP mRNA degradation in mammalian cells during ER stress. (A) RNA SLs from mouse Hgsnat, Blos1, and Xbp1. Red lettering indicates Xbp1 loop residues conserved across species, and arrows indicate putative Ire1 cleavage sites. Numbering is relative to mRNA translation start sites. (B–F) We stably transfected MC3T3-E1 cells with plasmids expressing reporter mRNAs, incubated cells with or without DTT (2 mM, 4 h), and measured relative abundances of the mRNA reporters by qPCR relative to the housekeeping control Rpl19. (B, C) Reporters expressing the mHgsnat (B) or mBlos1 (C) coding sequences (CDSs) with and without mutations in the Xbp1-like loop. (D) Reporters expressing the Blos1 CDS with and without mutations in the stem region of the Xbp1-like SL. Blue lettering indicates mutated residues. (E) Changes in mRNA abundance for the WT Blos1 reporter in two independent cell lines (WT1 and WT2) after DTT treatment. The cell lines differ only in their levels of reporter expression (Ex), either low (4-fold above endogenous levels) or high (32-fold above endogenous levels). (F) Reporters expressing GFP or an ER-targeted GFP (ssGFP) with and without the mBlos1 SL inserted 15 nt downstream of the stop codon. (G) Fraction membrane (membrane/total) of mRNAs from MC3T3-E1 cells stably expressing different GFP reporters measured by digitonin fractionation followed by qPCR. (H) We depleted Ire1 from stably transfected cells and then measured reporter mRNA levels as in B–F. Shown are averages and SDs from three or more independent experiments. *$p < 0.05$, two-tailed unpaired *t* test. Ut, untreated.

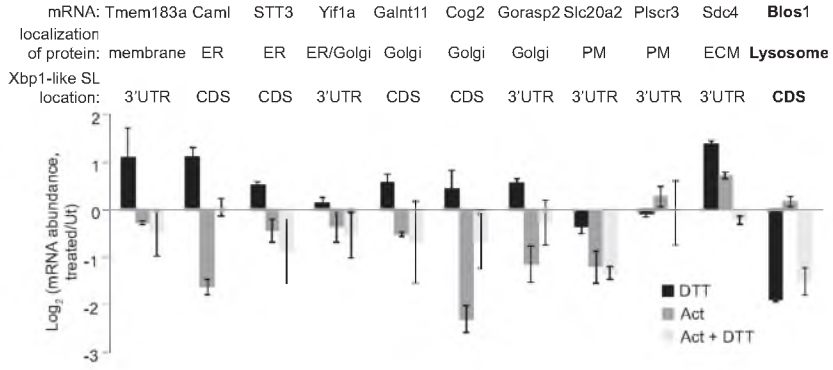

FIGURE 3: Xbp1-like SLs are not sufficient to target endogenous mRNAs to RIDD. MC3T3-E1 cells were treated with 1 mM DTT, 2 µg/ml actinomycin D (Act), or both for 4 h. We then measured relative mRNA levels of noted transcripts by qPCR. Transcripts were chosen based on their predicted localization to the ER (based on Gene Ontology term analysis) and the presence of Xbp1-like SLs, defined as 1) a seven-membered loop with the four conserved residues (as in Figure 2A), and 2) a stem of at least 5 base pairs including three GC pairs. The verified RIDD target Blos1 was also measured as a control. Shown are averages and SDs from two independent experiments. ECM, extracellular matrix; PM, plasma membrane; Ut, untreated.

Perk plays a role in the mammalian RIDD pathway, we transfected MC3T3-E1 cells with either a negative control (Neg) siRNA or a combination of four siRNAs targeting Perk and then induced ER stress with either DTT or Tg. Depletion of Perk strongly inhibited RIDD of both Blos1 and Col6a1 and partially inhibited RIDD of Scara3 (Figure 4, A–C). RIDD of Hgsnat, however, was not affected by Perk knockdown (Figure 4B; see next section). We saw similar effects when two distinct Perk siRNAs were transfected individually (Supplemental Figure S1, A and B). Finally, Perk knockdown also inhibited RIDD of Blos1 in Hek293 cells (Figure 4, D and E), indicating a conserved effect across species.

In addition to phosphorylating eIF2α and thereby attenuating translation initiation, Perk also phosphorylates other targets, including Nrf2 (Cullinan *et al.*, 2003) and diacylglycerol (Bobrovnikova-Marjon *et al.*, 2012). To determine which aspect of Perk signaling is important for RIDD, we used integrated stress response inhibitor (ISRIB), a chemical that blocks translation attenuation during ER stress but does not affect the phosphorylation of eIF2α or other Perk targets (Sidrauski *et al.*, 2013). ISRIB significantly inhibited RIDD (Figure 4F and Supplemental Figure S1C). Therefore Perk's ability to attenuate translation during ER stress is important for RIDD. Accordingly, artificially attenuating translation with the initiation inhibitor harringtonine fully restored RIDD in cells depleted of Perk (Figure 4G).

Knockdown of Perk also resulted in a 25–40% decrease in Xbp1 splicing in response to ER stress (Figure 4H), an effect noted previously (Majumder *et al.*, 2012). As with RIDD, inhibition of translation initiation by harringtonine fully restored Xbp1 splicing. Harringtonine did not cause a general increase in Ire1 activity, as harringtonine treatment alone actually led to a reduction in constitutive Xbp1 splicing in unstressed cells (Figure 4H). Overall these results indicate that attenuating translation initiation during ER stress allows for more efficient RIDD and Xbp1 splicing.

RIDD relies on the translational status of target mRNAs

There are two general possibilities for why Perk-mediated translation attenuation is important for RIDD: either halting translation allows for depletion of an unstable factor that is important for RIDD, or depletion of ribosomes from the RIDD target mRNA allows it to be degraded. The fact that Perk knockdown had varying effects on different mRNAs suggests that translation attenuation of the RIDD target itself is of primary importance. In support of this model, we noted that Hgsnat, the RIDD target that was insensitive to Perk depletion, has two large clusters of rare codons near the 5′ end of the transcript, which may act to constitutively reduce translation and allow for Hgsnat mRNA degradation during ER stress, regardless of Perk activity. Rare codon clusters were not found in the 5′ regions of Perk-sensitive RIDD targets (see later discussion of Figure 7A).

To test directly whether the translational status of mRNA targets is important for RIDD, we asked whether limiting translation of Perk-sensitive RIDD targets caused them to become Perk insensitive. We introduced translation-stalling SLs (Vattem and Wek, 2004) 6 nt upstream of the translation start site within the 5′ UTRs of two RIDD reporters, one expressing the Blos1 CDS (as in Figure 2C) and one expressing ssGFP with the Blos1 SL inserted in the CDS, 68 nt upstream of the stop codon (ssGFP-SL_{CDS}). We then stably transfected these reporters into MC3T3-E1 cells and tested for RIDD as described. In Neg siRNA–treated cells, these reporter mRNAs were degraded similarly to their wild-type counterparts. However, unlike the wild-type reporters, degradation of the translationally stalled reporters was unaffected by depletion of Perk (Figure 5, B and C). We conclude that attenuating translation of the target itself is important for degradation by RIDD.

Ribosome binding to an mRNA may limit Ire1's access, thus inhibiting cleavage and subsequent degradation of the mRNA. To test this idea we used cycloheximide (Chx), a translation elongation inhibitor that stalls ribosomes along mRNAs without releasing them. Chx significantly inhibited RIDD of both Blos1 and Col6a1 but not Scara3 (Figure 5D), correlating with the relative sensitivities of these mRNAs to Perk depletion. These results indicate that attenuating translation initiation and essentially reducing the number of ribosomes on an mRNA enhances RIDD, whereas blocking translation elongation by locking ribosomes on an mRNA inhibits RIDD.

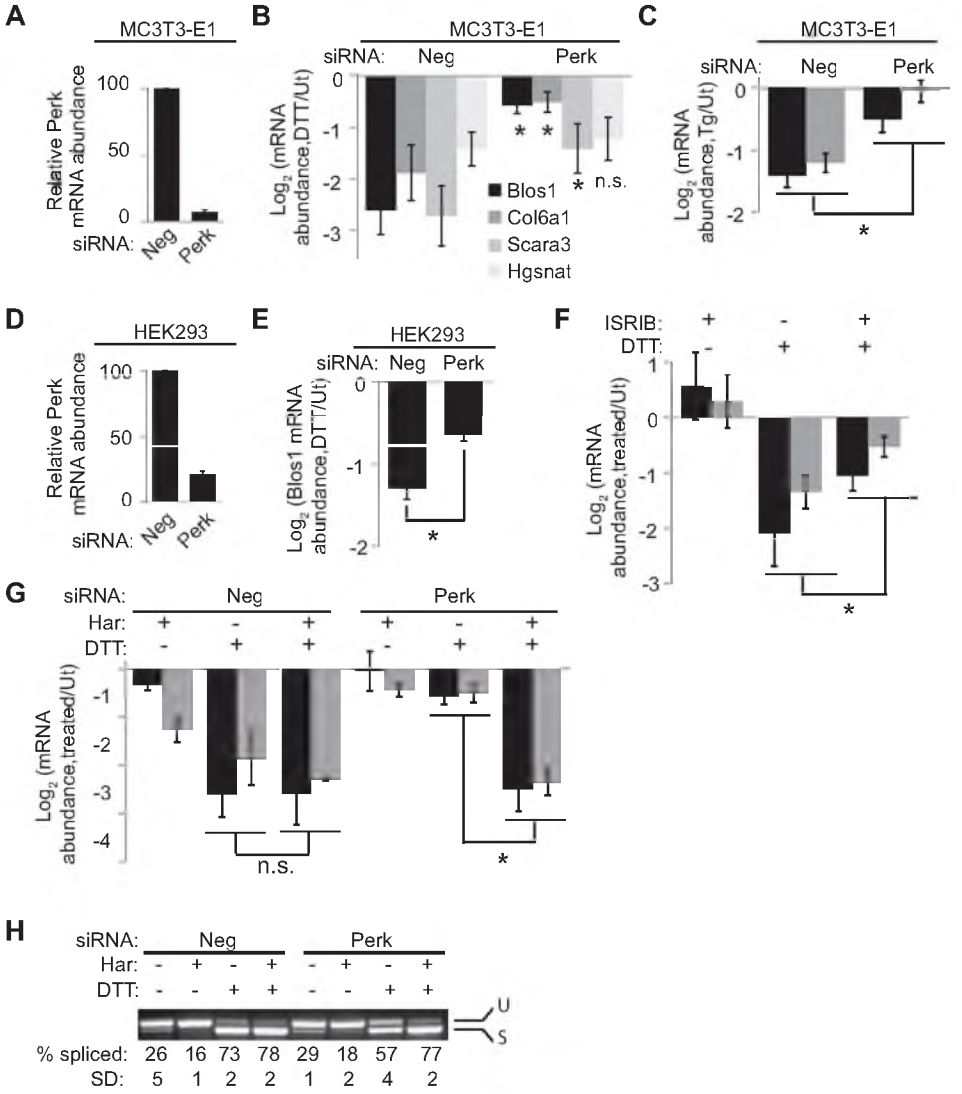

FIGURE 4: Translation attenuation mediated by Perk is important for RIDD. (A–C) We transfected MC3T3-E1 cells with Neg or Perk siRNAs and then incubated them with and without 1 mM DTT (B) or 2 μM Tg (C) for 4 h. We then measured the percentage of Perk mRNA remaining (A) and RIDD target mRNA levels (B, C). The legend in B applies to bar graphs in B, C, F, and G. Asterisks represent significant differences between Neg and Perk siRNA-treated samples. (D, E) Perk (D) or human Blos1 (E) mRNA measured from Neg or Perk siRNA–treated Hek293 cells with or without DTT (2 mM, 4 h). (F) Blos1 (black bars) and Col6a1 (gray bars) mRNA levels in MC3T3-E1 cells treated with 500 nM ISRIB, 1 mM DTT, or both for 4 h. (G, H) Blos1 (black bars) and Col6a1 (gray bars) mRNA levels (G) and Xbp1 splicing (H) from control or Perk-depleted MC3T3-E1 cells treated with 1 μM harringtonine (Har), 1 mM DTT, or both for 4 h. All mRNA levels were determined by qPCR. Shown are averages and SDs from at least three independent experiments. *$p < 0.05$, two-tailed paired t test. Ut, untreated.

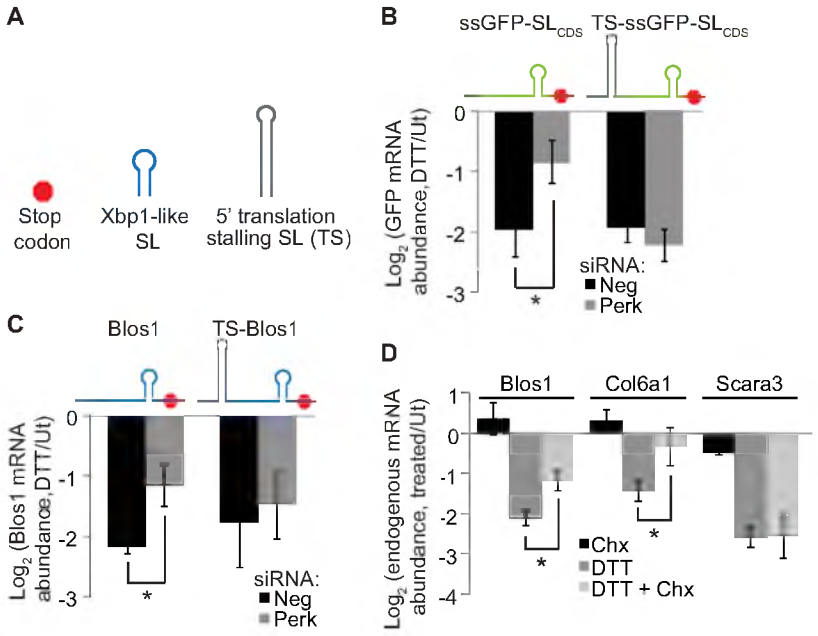

FIGURE 5: RIDD relies on the translational status of target mRNAs. (A) Legend for the diagrams. (B, C) We stably transfected MC3T3-E1 cells with plasmids expressing reporter mRNAs and then transfected them with Neg or Perk siRNAs and incubated cells with or without DTT (2 mM, 4 h). Reporters express ssGFP-SL_{CDS} (B) or mBlos1 (C) with or without upstream translation-blocking SLs inserted into their 5′ UTRs 6 nt upstream of the start codons. mRNA levels were measured by qPCR with reporter-specific primers. (D) Endogenous RIDD targets from MC3T3-E1 cells treated with 35 µM Chx, 1 mM DTT, or both for 4 h. Shown are averages and SDs from at least three independent experiments. *$p < 0.05$, two-tailed paired *t* test. Ut, untreated.

Translation attenuation of Xbp1-like SLs is important for RIDD

Based on the evidence that Ire1 directly cleaves RIDD targets in their Xbp1-like SLs, we wondered whether reduced ribosome occupancy in this specific region, rather than the entire message, is important for RIDD. We devised two strategies to test this hypothesis. First, we predicted that RIDD targets with Xbp1-like SLs in the CDS would be sensitive to Perk depletion, whereas RIDD targets with SLs in the 3′ UTR would be insensitive to Perk. As noted, degradation of the ssGFP-SL_{CDS} reporter during ER stress was reduced when Perk was depleted (Figure 5B). In contrast, the ssGFP-SL_{UTR} reporter, which has a stop codon 15 nt upstream of the Xbp1-like SL, was not sensitive to Perk knockdown (Figure 6B). Because these two constructs differ only in the presence of the upstream stop codon, the overall translation of the two constructs should be the same. Thus, translation of the Xbp1-like SL region appears to strongly influence whether a RIDD target will be affected by Perk.

We observed a similar trend for reporters expressing the Blos1 CDS. To determine whether moving Blos1's SL would affect its Perk dependence, we used the Blos1 reporter containing the G360C loop mutation, which is not degraded during ER stress (Figure 2C). We inserted a functional Xbp1-like SL (from wild-type Blos1) at an alternative position in the CDS of the mutated Blos1 reporter (position 261 relative to the translation start) or in the 3′ UTR (21 nt downstream of the stop codon). Moving the SL within the Blos1 CDS did not change the Perk sensitivity of the reporter (Figure 6C). Moving the SL to the 3′ UTR resulted in a decrease in overall degradation; however, this degradation was not affected by Perk depletion (Figure 6C).

In a second approach to test the importance of translation in the SL region, we used ribosome-stalling pseudoknots with different structures and sequences from either the infectious bronchitis virus (IBV) or simian retrovirus-1 (SRV-1; Kontos *et al.*, 2001). We introduced these sequences 15 nt upstream of the Xbp1-like SL in the CDS of our Blos1 reporter, preserving the original reading frame. Conveniently, the endogenous Blos1 SL is located within 30 nt of the stop codon, meaning that the majority of the Blos1 mRNA was translated normally with or without the pseudoknots. Both of these reporter mRNAs were degraded during ER stress similarly to the wild-type Blos1 reporter; however, Perk knockdown did not affect degradation of either pseudoknot-containing mRNA (Figure 6D). Differences in the efficiency of Perk depletion did not account for these effects, as endogenous Blos1 measured by qPCR with primers that amplified the endogenous CDS and 3′ UTR but not the reporter 3′ UTR, was equally sensitive to Perk knockdown in all cell lines tested (Supplemental Figure S1D). These data suggest that translation attenuation of only the SL region of the RIDD target is required for degradation.

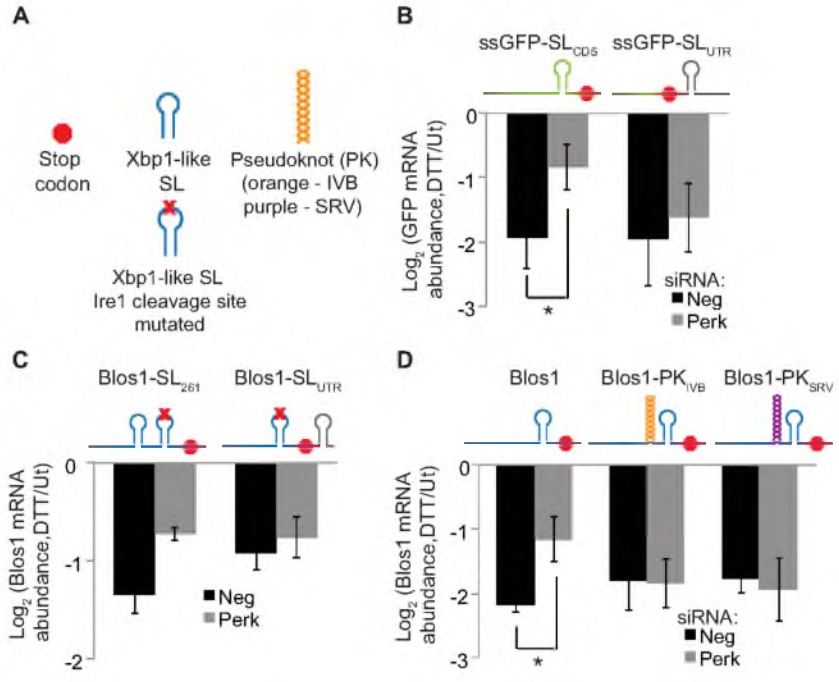

FIGURE 6: Translation attenuation of Xbp1-like SLs is required for RIDD. (A) Legend for the diagrams. (B–D) We stably transfected MC3T3-E1 cells with plasmids expressing reporter mRNAs and then transfected them with Neg or Perk siRNAs and incubated cells with or without DTT (2 mM, 4 h) as in Figure 5. (B) Reporters expressing ssGFP-SL_{CDS} or ssGFP-SL_{UTR}. (C) Reporters expressing RIDD-insensitive Blos1 containing the G360C loop mutation, with a functional Xbp1-like SL (from wild-type Blos1) added to the CDS at nt 261 (two independent experiments) or to the 3′ UTR. (D) Reporters expressing the mBlos1 CDS with or without the IBV pseudoknot (orange) or SRV pseudoknot (purple) inserted 15 nt upstream of the Xbp1-like SL. For B–D, we measured relative mRNA abundances by qPCR using reporter-specific primers. Shown are averages and SDs from at least three independent experiments except where noted. *$p < 0.05$, two-tailed paired t test. Ut, untreated.

DISCUSSION

In response to ER stress, the nuclease activity of Ire1 has two outputs. One is to initiate the splicing of the Xbp1 mRNA, leading to the transcriptional regulation of a large number of target genes. The second is to initiate the degradation of RIDD targets. Although these two outputs can be uncoupled (Han *et al.*, 2009; Hollien *et al.*, 2009), the mRNA sequence elements important for cleavage of RIDD substrates in mammalian cells are remarkably similar to those important for cleavage of the Xbp1 mRNA, namely stable SL structures with specific, conserved loop residues. However, despite the apparent sufficiency of such a SL in targeting GFP to the RIDD pathway (Figure 2F), there are many mRNAs in the cell that possess Xbp1-like SLs but are not targeted to RIDD (Figure 3; Bright *et al.*, 2015). We suggest that this additional specificity arises in part from the translational status of would-be target mRNAs, which we propose influences the accessibility of the Xbp1-like SLs.

A large body of evidence supports a role for translation in dictating an mRNA's susceptibility to degradation (Roy and Jacobson, 2013; Walters and Parker, 2014), and it appears that this is true for RIDD as well. We show here that RIDD in mammalian cells relies on Perk-mediated attenuation of translational initiation during ER stress, in a substrate-specific manner. Two RIDD targets with Xbp1-like SLs in their CDSs (Blos1 and Col6a1) were highly sensitive to knockdown of Perk, which blocked their degradation during ER stress. A third RIDD target (Scara3), with an Xbp1-like SL immediately upstream of its start codon, was partially sensitive to Perk depletion. The translation elongation inhibitor Chx inhibited RIDD of Blos1 and Col6a1 but not of Scara3. This is consistent with the idea that it is translation of the SL region that is important, as elongation inhibitors should not affect the small ribosomal subunit while it is scanning the 5′ UTR. Finally, Hgsnat, a RIDD target with natural clusters of rare codons (one at the 5′ end of the CDS and one immediately upstream of the Xbp1-like SL), was completely insensitive to Perk depletion, suggesting that it is normally translated at a low enough level to allow for RIDD in the absence of further translational attenuation (Figure 7A). Artificially stalling translation of RIDD reporter mRNAs extended these observations, as mRNAs containing

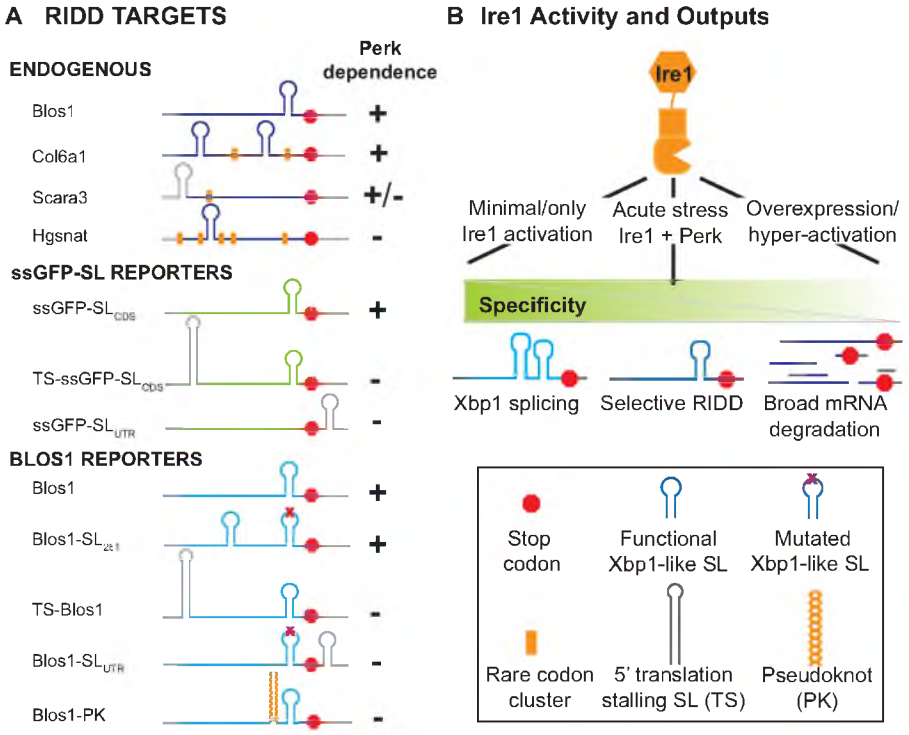

FIGURE 7: RIDD target summary and model. (A) Summary of Perk-dependent RIDD targeting for endogenous and reporter mRNAs. RNA CDSs are shown in color; UTRs are in gray. Diagrams are the same as in Figures 5 and 6. Orange bars denote clusters of rare codon usage, defined as >10% usage of infrequent codons over multiple 18-codon groupings as calculated by the Rare Codon Calculator (Clarke and Clark, 2008). (B) Model of differential Ire1 targeting, with legend of diagrams used.

translation-stalling SLs, pseudoknots, or stop codons upstream of their Xbp1-like SLs were insensitive to Perk depletion (Figure 7A).

Our data support a model in which attenuation of translation in mammalian cells, mediated by Perk or by natural sequence elements, leads to the formation of an accessible Xbp1-like SL in a target mRNA, which is then cleaved by Ire1 to initiate degradation. When Perk is depleted and translation is allowed to proceed, ribosomes would be expected to disrupt the secondary structure of the Xbp1-like SL as they move through this region. This effect, combined with ribosome physical occupancy of the mRNA, would limit the ability of Ire1 to access and cleave the target mRNA. These same mechanisms may also apply to Xbp1, as Xbp1 splicing was reduced when Perk was depleted and rescued by the addition of the translation initiation inhibitor harringtonine (Figure 4H). Although Perk depletion did not broadly inhibit RIDD in *Drosophila* S2 cells, target mRNAs are likely sensitive to translation, as continued, high levels of translation during ER stress can protect certain transcripts from RIDD (Gaddam *et al.*, 2013). In S2 cells, however, Xbp1-like SLs are not required for RIDD, and thus ribosomes may sterically hinder Ire1 access to the mRNA in a more general manner.

This coordination between Perk-mediated translation attenuation and Ire1 cleavage of mRNAs may tailor the UPR to specific types of stress (Figure 7B). UPR sensors are activated differentially under distinct forms of ER stress, and the involvement of Perk may limit RIDD to cases of ER stress in which both Ire1 and Perk are activated, such as hypoxia (Koumenis *et al.*, 2002; Drogat *et al.*, 2007). Furthermore, this requirement would ensure that inappropriate activation of the RIDD pathway does not occur in cases of stress in which only Ire1 is activated, such as plasma cell differentiation (Ma *et al.*, 2010). In addition, Perk initiates a negative feedback loop via induction of the phosphatase GADD34, which dephosphorylates eIF2α and restores protein translation (Novoa *et al.*, 2001; Ma and Hendershot, 2003). Through this mechanism, Perk may temporally limit robust degradation of RIDD targets.

Our data indicate that in mammals, RIDD is much more selective than in flies and suggest that the specific targeting of particular mRNAs is important for ER stress recovery. However, when Ire1 is overexpressed or otherwise hyperactivated, the requirements for both Perk and Xbp1-like SLs are lost (Han *et al.*, 2009; So *et al.*, 2012), suggesting that mammalian Ire1 is capable of a much broader specificity in certain circumstances (Figure 7B). We speculate that

broad cleavage of mRNAs may occur in mammalian cells exposed to acute ER stress as well but to a small degree such that the steady-state levels for most mRNAs do not measurably change. This activity may be important in the local control of ER load or in the response to viruses, in which Rig-I has recently been shown to be activated by the products of RIDD (Cho *et al.*, 2013).

MATERIALS AND METHODS

Cell culture/ER stress

We cultured MC3T3-E1 cells (American Type Culture Collection, Manassas, VA) in MEMα with nucleosides and no ascorbic acid (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and antibiotics unless otherwise stated. Min6, Hek293, and Hep G2 cells (from J. Rutter, A. V. Mariqc, and C. H. Hagedorn, respectively, University of Utah) were cultured in DMEM (Invitrogen) supplemented with 10% FBS, antibiotics, and Gln. All cell lines were maintained at 37°C and 5% CO_2. We added 1 or 2 mM DTT (Sigma-Aldrich, St. Louis, MO), 2 μM Tg (Sigma-Aldrich), or 2 μg/ml Tm (EMD Millipore, Billerica, MA) to cell media for 4 h to induce ER stress. For inhibition of transcription or translation elongation, we added 2 μg/ml ActD (Sigma-Aldrich) or 35 μM Chx (Sigma-Aldrich), respectively, to cells for ~5 min before adding 1 mM DTT. ISRIB was a kind gift from the Peter Walter lab (University of California at San Francisco, San Francisco, CA). For ISRIB experiments, we added 500 nM ISRIB to cells for ~5 min before adding DTT.

siRNA

For Ire1, Xbp1, and Perk siRNA experiments, we cultured cells in antibiotic-free media. We followed published RNAiMAX (Invitrogen) protocols to transfect cells with organism-specific siRNAs (Qiagen, Valencia, CA). The following siRNA reagents were used: Ire1 (SI00995890, 897, 904), Xbp1 (GS22433), Perk combined (GS1366-mouse or GS9451-human), Perk #1 (SI00991319), and Perk #3 (SI00991333). We controlled for the effects of the siRNA procedure by including Neg siRNA (Qiagen)–transfected samples in all experiments. We incubated cells for 48–72 h before replacing media and treating with or without ER stress. For harringtonine experiments, we added 1 μg/ml harringtonine (LKT Laboratories, St. Paul, MN) to media in Neg and Perk siRNA–treated cells and incubated for ~5 min before the addition of DTT.

Plasmid reporter construction and transfection

For wild-type Blos1 and Hgsnat reporters, we amplified the Blos1 (UniGene ID Mm.30118) or Hgsnat (UniGene ID Mm.28326) CDS from MC3T3-E1 cell cDNA and subcloned downstream of the human EF-1α promoter. To create plasmids expressing our reporter mRNA and a hygromycin resistance gene, we then subcloned the promoter and CDS between the *Nru*I and *Not*I sites of the pcDNA3.1-Hygro(+) vector (Invitrogen). For our GFP and ssGFP reporters, we subcloned the GFP or ssGFP sequences previously described (Gaddam *et al.*, 2013) into our expression vector. To create

RNA	Primer 1	Primer 2
mBlos1 (endogenous)	CAAGGAGCTGCAGGAGAAGA	CCAGGAGGGTGAAGTAAGAGG
mScara3	TGCATGGATACTGACCCTGA	GCCGTGTTACCAGCTTCTTC
mCol6a1	TGCTCAACATGAAGCAGACC	TTGAGGGAGAAAGCTCTGGA
mHgsnat (endogenous)	TCTCCGCTTTCTCCATTTTG	CGCATACACGTGGAAAGTCA
hBlos1	CAAGGAGCTGCAGGAGAAGA	GCCTGGTTGAAGTTCTCCAC
hScara3	AACTTCCTGCACACACTGGC	CAAACCAGTTGCACATCCAG
mXbp1	AGAAGAGAACCACAAACTCCAG	GGGTCCAACTTGTCCAGAATGC
hXbp1	AGCTCAGACTGCCAGAGATCG	AATCCATGGGGAGATGTTCTA
mHgsnat (reporter)	GGAACCCCCTTCTTCTATCC	GGAAAGGACAGTGGGAGTGG
mBlos (reporter)	CAAGGAGCTGCAGGAGAAGA	GGAAAGGACAGTGGGAGTGG
GFP	TAATACGACTCACTATAGGGAGA	TGCTCAGGTAGTGGTTGTC
mGapdh	TGAACGGGAAGCTCACTGG	GGTCCTCAGTGTAGCCCAAG
mBip	TCAGCATCAAGCAAGGATTG	AAGCCGTGGAGAAGATCTGA
mSdc4	AACTGAGGTCTTGGCAGCTC	TCCCCAATAAGTCCAAGCAG
mCaml	GCGAGAAGAAGGTGAAGACG	TAAGTTCCTCGGGTTTCAGG
mTmem183a	CTCTTTGACTGGTGGCATCC	TTCAACCTTTCCACCTCCTG
mGalnt11	ATGGCTCCTCCTCTCAACAG	CAGCAGCTCGGAAGTAAACC
mGorasp2	CGAGAAGCCTGTGTCTGATG	CAGCCTCTTGCGTAGTTTCC
mSTT3	CATCGTCCCCAAACAGAAGT	TGTACCCTTGGTGCTGTGAA
mCog2	GGAGACGGTCAAGCAGAAAC	TATTGGTCCTGCGGTAAAGC
mYif1a	CCAAGGGAAGGACATAGTGC	TAGAGGTCAGGGGCATTGAG
mPlscr3	GGCATCCCTTCCTTCCTAAG	CAAAGTCATCGGCATCTGTG
mSlc20a2	GTTGCATCTTCCCATTGCTT	GACACCGAGTGGGACTTGAT
mBlos1 (endogenous plus reporter)	CCAGGCCTACATGAACCAGA	TAGACGTATTCCAGCGCAGT

TABLE 1: Primers used for qPCR and Xbp1 splicing measurements (5′ to 3′).

GFP-SL_{UTR} and ssGFP-SL_{UTR} reporters, we added 33 nt from the 3′ end of the Blos1 CDS, which includes the Xbp1-like SL sequence, 15 nt downstream of the GFP or ssGFP CDS. We introduced site-directed mutations in reporters by fusion PCR and translation-stalling SLs (Vattem and Wek, 2004) or pseudoknots (Kontos *et al.*, 2001) in reporters from Figures 5 and 6 by oligo cassette mutagenesis. We inserted pseudoknot sequences 15 nt upstream of the Blos1 SL.

For all reporters, we created polyclonal stable cell lines by transfecting 2 µg of plasmid into MC3T3-E1 cells using Lipofectamine 2000 (Invitrogen). We replaced media after 1.5–2 h and allowed cells to recover for an additional 24–36 h before passaging and adding 100 µg/ml hygromycin B. Hygromycin B–resistant cells were selected over a 2- to 3-wk period and cultured in 100 µg/ml hygromycin B thereafter. For reporter assays, cells were passaged into hygromycin-free media 48–72 h before treatment with or without 2 mM DTT for 4 h.

Digitonin fractionation

We used a modified procedure based on a protocol developed by Stephens *et al.* (2008) for separation of cytosolic and membrane-bound mRNAs. Briefly, we incubated MC3T3-E1 cells with 35 µM Chx for 10 min and then trypsinized and pelleted cells. We resuspended cells in cytosol buffer (150 mM KOAc, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 2.5 mM $Mg(OAc)_2$, 200 U/ml RNaseOUT, 35 µM Chx). We immediately permeabilized cells with 1 mg/ml digitonin and incubated on ice for 15 min. Cell lysates were then centrifuged at 500 × *g* for 5 min, and supernatant was collected as the cytosolic fraction. We resuspended the pellet in cytosol buffer with 1% Triton X-100, and incubated and centrifuged as before. The supernatant was collected as the membrane-bound fraction.

mRNA isolation and analysis

We isolated mRNA using either TRIzol reagent (Invitrogen) or Zymo Research Quick-RNA MiniPrep kits. We then synthesized cDNA using 1–2 µg of total RNA as template, a T18 primer, and MMLV reverse transcriptase (NEB, Ipswich, MA). We measured relative mRNA levels by qPCR using the Masterplex ep realplex (Eppendorf, Hauppauge, NY) with SYBR green fluorescent dye. Each sample was measured in triplicate, and target mRNA levels were normalized to those of ribosomal protein 19 (Rpl19) mRNA. To ensure that signal was not due to contaminating plasmid or genomic DNA, we also measured mRNA levels from samples to which no reverse transcriptase was added.

For specifically detecting mRNA expressed from reporters, we used one primer designed to bind the reporter CDS and one primer designed to bind the 3′ or 5′ UTR derived from the vector. These primers did not amplify endogenous transcripts, which we tested using untransfected cells or cells transfected with control reporters (e.g., GFP).

We quantified Xbp1 splicing by amplifying cDNA with primers that surround the Xbp1 splice site and running the products on a 2% agarose gel. Relative band intensities for the spliced and unspliced products were quantified using ImageJ (National Institutes of Health, Bethesda, MD).

All primer sequences are shown in Table 1.

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Supplemental Materials

Molecular Biology of the Cell

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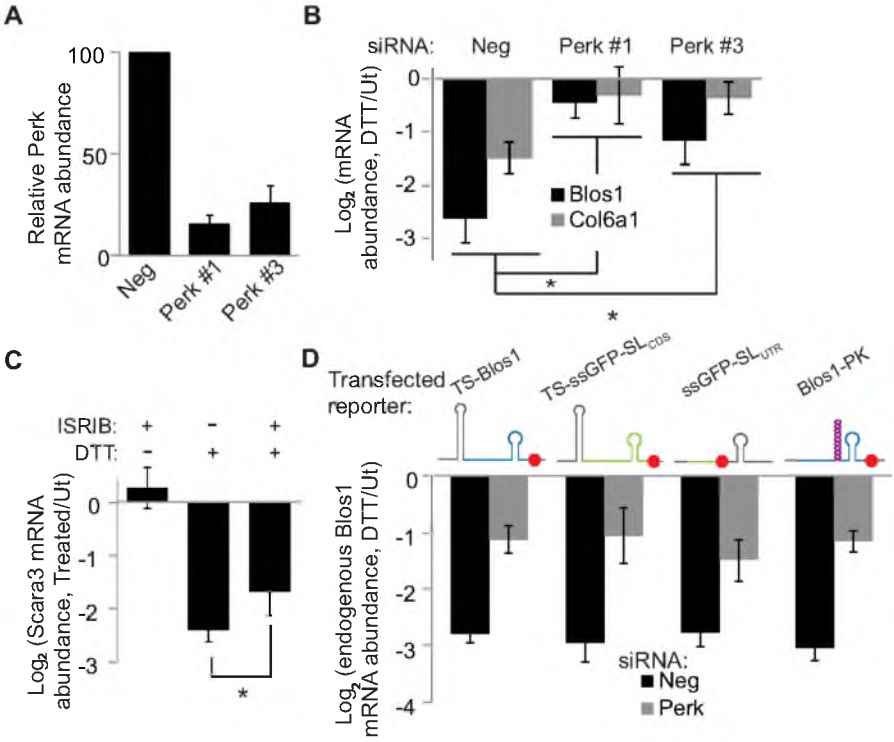
A
Relative Perk mRNA abundance
100
50
0
Neg
Perk #1
Perk #3
B
siRNA: Neg Perk #1 Perk #3
Log2 (mRNA abundance, DTT/Ut)
0
-1
-2
-3
Blos1
Col6a1
*
*
C
ISRIB: + - +
DTT: - + +
Log2 (Scara3 mRNA abundance, Treated/Ut)
0
-1
-2
-3
*
D
Transfected reporter:
TS-Blos1
TS-ssGFP-SLCDS
ssGFP-SLUTR
Blos1-PK
Log2 (endogenous Blos1 mRNA abundance, DTT/Ut)
0
-1
-2
-3
-4
siRNA:
Neg
Perk

Supplemental Figure 1. (A-B) We transfected MC3T3-E1 cells with two different siRNAs targeting the Perk transcript (Perk #1 or Perk #3). We measured percent of Perk mRNA remaining (A) and RIDD target mRNA levels (B) after incubation with or without 1 mM DTT for 4 h. (C) Scara3 relative mRNA abundance in MC3T3-E1 cells treated with 500 nM ISRIB, 1 mM DTT, or both for 4 h. Samples are identical to those in Figure 4F. (D)Relative mRNA abundance of endogenous Blos1 from stably transfected cell lines expressing Perk-insensitive RIDD reporters. Samples are identical to those used to measure the reporter mRNA levels in Fig. 5C, 5B, 6B, and 6D. Shown are averages and standard deviations of at least 3 independent experiments. *$p<0.05$, two tailed paired t-test.Ut, untreated.

CHAPTER 4

LYSOSOME ACCUMULATION DURING ER STRESS

Introduction

The physiological function of regulated Ire1-dependent decay (RIDD) is not well understood. While prolonged activation of Ire1 due to over expression or hyperactivation results in observable effects of RIDD on cell biology, many of these effects appear to be cell type and organism dependent (So *et al.*, 2012; Benhamron *et al.*, 2014). Our goal in this project was to determine whether a ubiquitous function of RIDD exists, and how it may influence the cellular response to various insults of ER stress. A recent analysis of RIDD targets from multiple data sets, across a variety of cell types and organisms indicated that the only mRNA consistently targeted to the RIDD pathway is Blos1 (Bright *et al.*, 2015). For this reason we decided to determine whether specific loss of Blos1 mRNA through the RIDD pathway could contribute to the ER stress response.

The Blos1 protein was originally characterized as part of the biogenesis of lysosome-related organelle complex 1 (BLOC1), which is essential for biogenesis of lysosome related organelles (LROs) such as melanosomes and platelet-dense granules (Dell'Angelica, 2004). Interestingly, while mutation or knockout of most BLOC1 components results in deficiency of LRO biogenesis and phenotypic changes such as coat coloration defects (Dell'Angelica, 2004), knockout of Blos1 in mice results in embryonic lethality (Scott *et al.*, 2014; Zhang *et al.*, 2014). Additionally, Blos1 has been observed in the mitochondria and may play a role in acetylation of mitochondrial proteins (Scott *et al.*, 2012; Scott *et al.*, 2014). Immunofluorescence and fractionation studies indicate that Blos1 partitions evenly between the cytosol and the mitochondria, but is not localized within any compartments of the endomembrane system (Ghaemmaghami *et al.*, 2003; Scott

et al., 2012). Recently, Blos1 along with two other BLOC1 components, Blos2 and Snapin, has also been characterized as a subunit of the BLOC-1-related complex (BORC) (Pu *et al.*, 2015). Depletion of the BORC complex components, including Blos1, results in perinuclear accumulation of lysosomes due to loss of kinesin-dependent anterograde lysosome transport (Pu *et al.*, 2015).

Perinuclear accumulation of lysosomes has been previously observed under various conditions including increases in intra- and extracellular pH, increases in cellular cholesterol, and cell starvation (Rocha *et al.*, 2009; Steffan *et al.*, 2009; Korolchuk *et al.*, 2011). While the consequences of lysosome repositioning are not fully understood, there is evidence that perinuclear accumulation of lysosomes results in increased likelihood of autophagosome-lysosome fusion and enhanced efficiency of autophagy (Korolchuk *et al.*, 2011). Increasing autophagic flux may allow cells to better compensate for various stresses (Murrow and Debnath, 2013). Conversely, more peripheral localization of lysosomes results in greater cell motility (Steffan *et al.*, 2010; Pu *et al.*, 2015).

Interestingly, both perinuclear accumulation of lysosomes and increases in autophagy have also been observed with ER stress (Deegan *et al.*, 2013; Elfrink *et al.*, 2013). While the function of lysosome accumulation during ER stress is unknown, increases in autophagy are hypothesized to induce bulk removal and degradation of misfolded proteins (Deegan *et al.*, 2013). Furthermore, increases in autophagosome-lysosome fusion have also been observed during ER stress (Raciti *et al.*, 2012). Based on these data, we propose a model in which loss of Blos1 mRNA via RIDD results in perinuclear accumulation of lysosomes and of

increased efficiency of autophagy, which augments the known cell survival pathways of the UPR.

Results

Lysosomes accumulate in the perinuclear region during ER stress

To determine whether lysosomes relocate to the perinuclear region during ER stress, we induced ER stress with thapsigargin (Tg) in cells expressing Lamp1-GFP, which marks lysosomes and late endosomes and is a common marker for lysosome localization (Eskelinen, 2006; Pu *et al.*, 2015). After 18hr treatment with Tg we saw a distinct shift in lysosome position toward the perinuclear region of cells (Figure 4.1, A and B). Because previous work has indicated that Ire1 activity (and thus Blos1 degradation) is attenuated at late time points during ER stress (Lin *et al.*, 2007), we also measured Blos1 mRNA levels and Xbp1 splicing at both 4 and 18hr after Tg treatment (Figure 4.1, C and D). Blos1 degradation was increased at 18hr compared to the 4hr time point (Figure 4.1C), and the level of Xbp1 splicing remained similar to that observed at 4hr (Figure 4.1D), indicating that at 18hr of Tg treatment Ire1 activity is undiminished. Finally, we determined the time scale of lysosome movement with ER stress. At 4hr of Tg treatment lysosome localization is indistinguishable from untreated cells (Figure 4.1, E and F). However, at 8hr of Tg treatment we see a shift towards perinuclear localization that continues to increase at 12hr of treatment (Figure 4.1, E and F). Because of the intermediate localization of lysosomes at the 8hr time point, these images were quantified using correlation values generated from

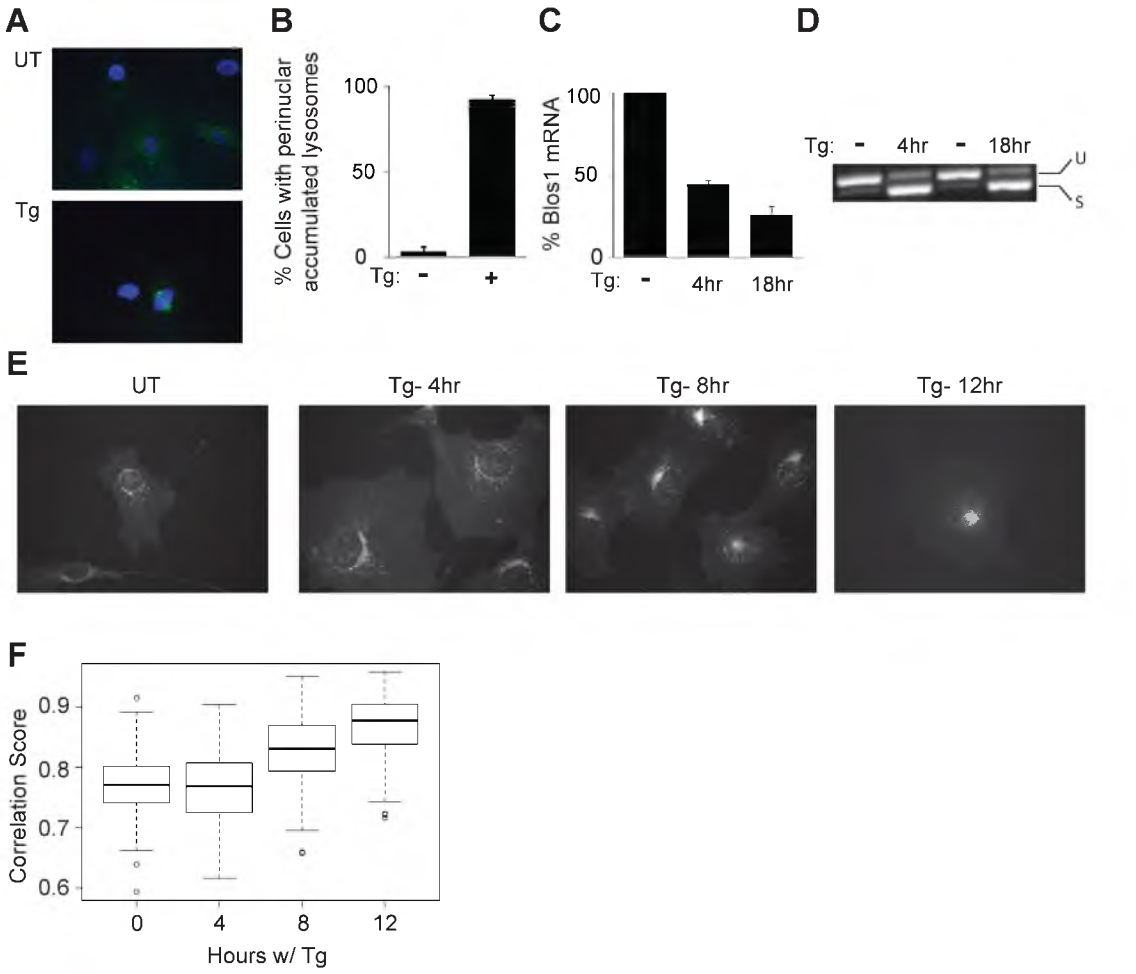

Figure 4.1. Lysosomes accumulate in the perinuclear region with ER stress. (A) Representative live cell images of MC3T3 cells stably transfected with Lamp1-GFP (green), and Hoechst dye to stain nuclei (blue) before and after 18hr Tg treatment. (B) Quantification of >100 images collected from 3 biological experiments analyzed for each condition (C) Relative Blos1 mRNA levels in MC3T3 cells treated with or without Tg (2uM) for either 4 or 12hr . (D) Samples from (C) amplified by PCR using primers surrounding the Xbp1 splice site. Shown are representative agarose gels with the spliced (s) and unspliced (u) products. (E) Representative live cell images of MC3T3 cells stably transfected with Lamp1-GFP with or without Tg (2uM) for noted time point. (F) Quantification of lysosome accumulation. > 100 images collected from 3 biological experiments analyzed from each time point.

the texture analysis module of the Cell Profiler software (Carpenter *et al.*, 2006). Higher correlation values are indicative of lysosome clustering (Figure 4.1F).

Lysosome movement during ER stress is Ire1-dependent and Xbp1-independent

To determine if RIDD could be involved in lysosome repositioning, we tested whether lysosome movement during ER stress was Ire1-dependent and Xbp1-independent. We treated Lamp1-GFP expressing cells with untargeted (Neg), Ire1, or Xbp1 siRNAs and assayed for perinuclear lysosome accumulation after 12hr of Tg treatment. Lysosomes relocated to the perinuclear region in either control or Xbp1 depleted cells. However, in cells treated with Ire1 siRNAs lysosome accumulation was diminished (Figure 4.2, A and B). To ensure that Ire1 knockdown effectively blocked degradation of Blos1 mRNA, we quantified Blos1 levels at various times after induction of stress. Interestingly, not only was Blos1 degradation significantly inhibited, but also the overall level of Blos1 mRNA was increased in Ire1 depleted cells (Figure 4.2B). We also confirmed Ire1 knockdown efficiency with Xbp1-splicing, and Xbp1 knockdown efficiency through qPCR (Figure 4.2, C and D).

Discussion

Taken together these results demonstrate that lysosome accumulation in the perinuclear region occurs in an Ire1-dependent, Xbp1-independent manner on a time scale consistent with continued Blos1 mRNA degradation. We hypothesize that the downstream effects of Blos1 mRNA degradation result from

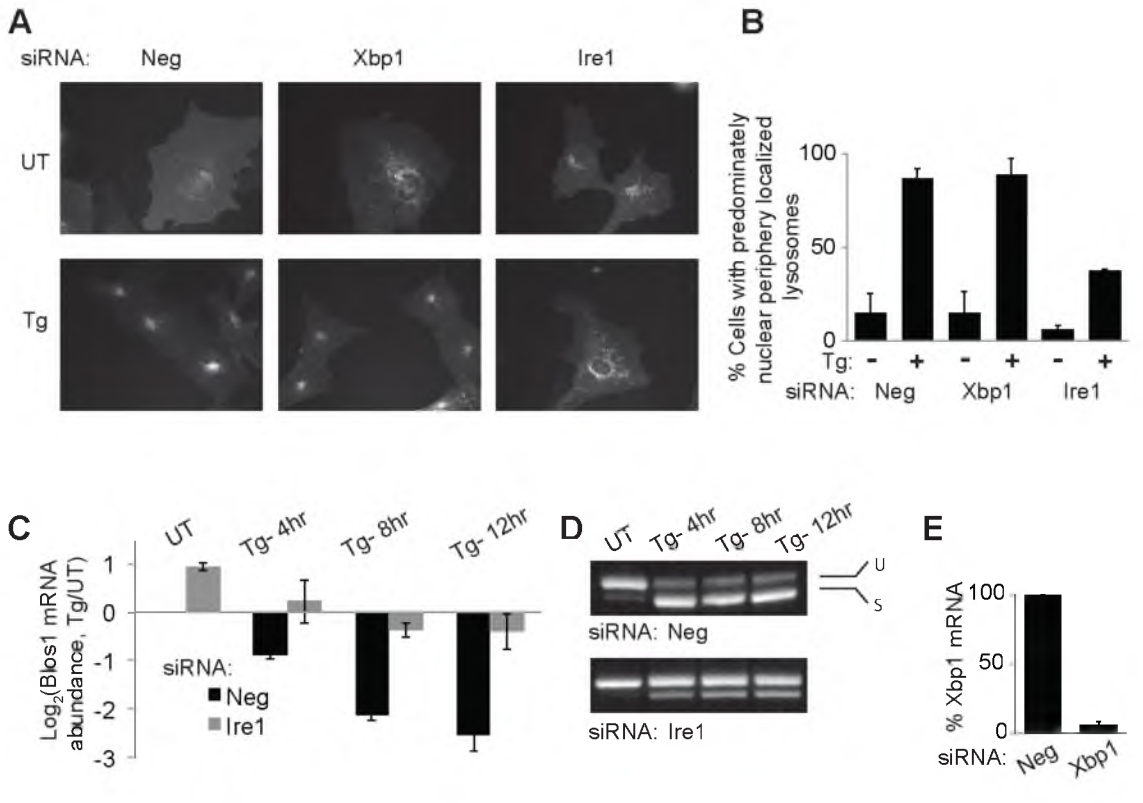

Figure 4.2. Lysosomal accumulation during ER stress is Ire1-dependent and Xbp1-independent. (A-E) MC3T3 cells stably transfected with Lamp1-GFP were treated with noted siRNAs for 24-48hr. (A) Representative live cell images of cells treated with Neg, Ire1, or Xbp1 siRNA before (UT- untreated) and after treatment with Tg (2uM) for 12hr. (B) Quantification of >150 images collected from 3 or more biological experiments analyzed for each condition. (C) Relative Blos1 mRNA levels from cells treated with Neg (Black bars) or Ire1 (gray bars) siRNA with and without Tg (2uM) for noted amount of time. mRNA levels normalized to Neg untreated (UT) sample. (D) Xbp1 splicing (as described in Figure 4.1) of samples from (B). (E) Relative Xbp1 mRNA levels from samples in (A).

a subsequent decrease in Blos1 protein levels. Thus for Blos1 mRNA degradation to have an effect on UPR signaling, the Blos1 protein must be relatively short-lived. A current analysis of protein stability within various compartments of the cell suggests that the cytoplasmic pool of Blos1 is relatively unstable (Larance *et al.*, 2013), indicating that Blos1 mRNA degradation in combination with translation attenuation mediated by Perk, could result in significant depletion of Blos1 protein over an 8-12hr timespan. These data support a model in which loss of Blos1 mRNA through RIDD leads to depleted Blos1 protein levels, resulting in inactivation of the BORC1 complex and repositioning of lysosomes (Figure 4.3). Alternatively, Jnk, which is activated in an Ire1-dependent, Xbp1-independent manner (Urano *et al.*, 2000) may also play a role in lysosome movement, although no evidence of Jnk-dependent lysosome movement has been reported.

Regardless of mechanism, perinuclear accumulation of lysosomes may be a significant factor in a cell's ability overcome ER stress. Numerous reports indicate that inhibiting autophagy during ER stress results in an increased rate of cell death (Bernales *et al.*, 2006; Ogata *et al.*, 2006). Increased accumulation of lysosomes in the perinuclear region has been shown to increase autophagic efficiency under starvation conditions, and may explain increases in autophagosome-lysosome fusion observed under ER stress conditions (Raciti *et al.*, 2012). This effect may have complicated implications for organism health as a whole. Increasing cellular resistance to stressful insults is often considered beneficial; however, in various cancer cell types, activation of the UPR and increases in autophagy both increase cell survival in tumor environments

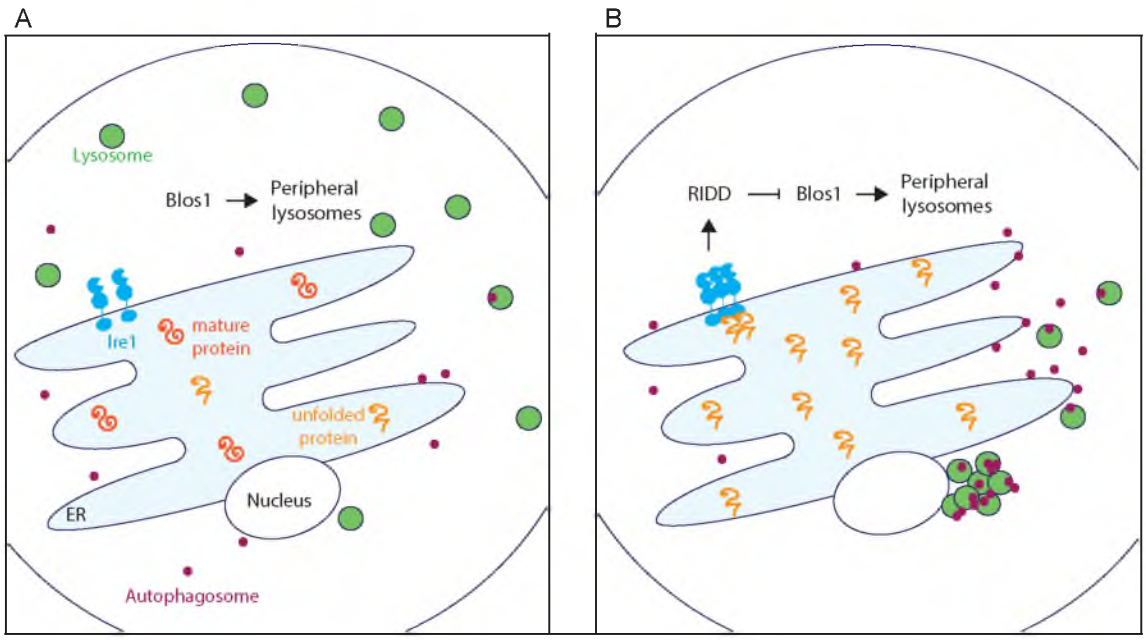

Figure 4.3 Model of Blos1-dependent lysosome accumulation during ER stress. (A) A cell in unstressed conditions. Blos1 is present. (B) A cell during ER stress. Active Ire1 cleaves Blos1 mRNA resulting in its degradation and subsequent depletion of the Blos1 protein. Loss of Blos1 inhibits the BORC complex and leads to lysosome accumulation, which enhances autophagosome-lysosome fusion and efficiency of autophagy during ER stress.

(Salazar *et al.*, 2011). Therefore, determining the pathways required for lysosome movement during ER stress will not only elucidate the mechanisms required for this phenomenon, but may also provide additional tools to specifically manipulate this system and create therapeutic targets.

Future Directions

To determine whether Blos1 mRNA degradation is responsible for the movement of lysosomes we have attempted multiple experiments to overexpress a noncleavable form of Blos1 mRNA in cells expressing Lamp1-GFP, and have thus far been unsuccessful in co-transfection. One concern that we face is that overexpression of Blos1 during ER stress may result in cell death; however because of low transfection efficiency of our Blos1 constructs it has been impossible to determine if this is the case. Current experiments using various Blos1 constructs and a lamp1 antibody are currently underway.

Although determining a physiological role for RIDD was the initial impetus for these experiments, if degradation of Blos1 does not induce lysosome movement, insights into other aspects of the ER stress response may still be gained from this project. As noted above, increased lysosome accumulation within the perinuclear region has been associated with increased autophagic flux (Korolchuk *et al.*, 2011), which appears to increase cell survival (Deegan *et al.*, 2014). To determine if lysosome repositioning observed during ER stress is consistent with this model, we are working towards assaying autophagic activity as well as autophagosome-lysosome fusion in cells lacking Ire1, which do not accumulate lysosomes in the perinuclear region at the same rate as wildtype

cells. It is well established that loss of Ire1 signaling results in increased levels of cell death. Thus to determine whether lysosome accumulation results in enhanced cell survival we must pursue other tools that inhibit lysosome accumulation during ER stress beyond loss of Ire1.

Materials and Methods

Cell culture/ER stress/siRNA

We cultured MC3T3 cells in MEM alpha media supplemented with 10% fetal bovine serum (FBS). To induce ER stress we added 2uM Tg to cells for varying time points. For Xbp1, Ire1, and Neg control siRNA experiments, we followed published RNAi max (Invitrogen) protocols to transfect MC3T3 cells with mouse specific siRNAs or untargeted control (Neg) siRNA. We incubated cells with siRNAs for 24-48hr before treatment with Tg.

Generation of Lamp1-GFP plasmid and stable cell line

We amplified Lamp1 (Mm.16716) from MC3T3 cDNA. We used PCR mutagenesis to mutate the endogenous stop codon and subcloned it upstream of GFP in our ssGFP expression vector described in Moore and Hollien 2015, which also contains a hygromycin resistance gene.

We transfected MC3T3 cells with the Lamp1-GFP plasmid following the transfection guidelines for Lipofectamine 2000 (Invitrogen). We incubated cells with the transfection mix for 2hr before replacing media. After 24hr, we split cells and added 100ug/mL hygromycin to select cells expressing the plasmid. Media was replaced every 3-4 days for a 2-week period while cells were selected for

hygromycin resistance. Thereafter we cultured stable cells in media containing 100ug/mL hygromycin.

mRNA isolation and analysis

For mRNA isolation and analysis we isolated RNA from MC3T3 cells with the Zymo RNA miniprep kit, and synthesized cDNA using 1ug of RNA, a T18 oligo, and MMLV reverse transcriptase. Relative Blos1 mRNA levels were measured by qPCR and normalized to mRNA levels of the housekeeping gene, ribosomal protein 19 (Rpl19).

Microscopy and image analysis

For live cell imaging, we placed cells expressing Lamp1-GFP on glass bottom plates in media without hygromycin for 24-48 hr. We imaged cells at room temperature on an Olympus IX51 microscope with 60X oil immersion objective at 0.5 seconds. For Figures 4.1A and 4.2A images were taken before and after Tg treatment. To avoid photo bleaching between image sets used in Figure 4.1E, two separate plates of cells were each imaged 2 or 3 times during the time course. To ensure that lysosome movement was not a function of the time cells spent in plates, we imaged untreated (UT) cells in one plate simultaneously with 12hr Tg treated cells from the second plate.

We used two independent methods to measure lysosome accumulation in the perinuclear region. First, an experimenter who was blind to cell treatment scored images of cells as either having predominantly perinuclear accumulated lysosomes or predominantly peripheral lysosomes. We generated the percent of

cells with predominantly perinuclear localized lysosomes for each treatment from 3 separate biological replicates to generate the data in Figure 4.1B and 4.2B. For Figure 4.1F, we analyzed lysosome accumulation with the Cell Profiler program. We loaded images into Cell Profiler and analyzed them with the Texture Measure module. Texture correlation values were averaged for all angles and used to create box plots.

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CHAPTER 5

SUMMARY AND CONCLUSION

Introduction

The UPR is comprised of pathways responsible for sensing the folding environment within the ER as well as generating the suitable response to various forms of ER stress. A cell's ability to appropriately respond to changes in secretion requirements is essential not only for cell function, but also for organism survival. Often the initial response to ER stress results in an increase in ER function and capacity; however, if stress is irremediable the UPR will initiate pro-apoptotic pathways (Moore and Hollien, 2012). These mechanisms are known to be essential for organism development (Reimold *et al.*, 2000; Zhang *et al.*, 2002; Yamamoto *et al.*, 2007; Iwawaki *et al.*, 2009) as well as responses to conditions such as pathogen infection (Iwakoshi *et al.*, 2003). However, there is growing evidence that aberrant activation of the UPR negatively affects organism health (Salazar *et al.*, 2011; Rabhi *et al.*, 2014). Interestingly both the prosurvival and pro-apoptotic pathways of the UPR have been implicated in enhanced disease progression. Thus understanding the mechanisms and functions of pathways induced by ER stress is essential to our ability to devise therapeutic treatments that effectively regulate the UPR while minimizing off target effects.

In an effort to better delineate the mechanisms and functions of Ire1-dependent UPR signaling I employed molecular biology techniques to investigate the regulated Ire1-dependent decay (RIDD) pathway. I initially studied a non-canonical RIDD target in S2 drosophila cells to better understand how exceptions to RIDD targeting might be informative to RIDD function in flies as well as mechanisms in other organisms such as mammals. While studying this pathway it became clear that noncanonical RIDD targeting in flies shared similarities to the

RIDD pathway observed in mammals. Through this work I was able to determine the sequence and structure requirements of mammalian RIDD targets, as well as a requirement for translational attenuation in the mammalian RIDD pathway. I have also gathered evidence that supports a functional model of RIDD that results in lysosome accumulation and possible effects on autophagic efficiency.

Noncanonical RIDD Targeting in Drosophila

The canonical model of RIDD targeting in Drosophila predicts that ER-localization is both necessary and sufficient for mRNA degradation; however RIDD targets have been identified that do not fit this model. The Sumo homolog in flies, Smt3, was identified as a potential RIDD target, although it lacked any sequence elements predicted to localize the mRNA to the ER (Hollien and Weissman, 2006). We show that the Smt3 transcript is not stably associated with the ER, and is targeted to RIDD via an alternative pathway. Degradation of Smt3 depends on an Xbp1-like stem loop structure in the coding region of the transcript as well as presence of Perk. We propose that translation attenuation mediated by Perk is required for cleavage of the Smt3 transcript. Although Perk is not generally required for RIDD in Drosophila, enhanced translation of ER-localized mRNAs during ER stress appears to protect them from the RIDD pathway (Gaddam *et al.*, 2013). These results indicate degradation of mRNA through the canonical RIDD pathway may be less sensitive to continued translation during ER stress. Alternatively, Perk-independent mechanisms of translation attenuation during ER stress exist (Garrey *et al.*, 2010), which may enhance the canonical RIDD pathway. Taken together these data describe both

the canonical and alternative mechanisms of RIDD targeting in *Drosophila* (Figure 5.1).

Mechanisms of RIDD Targeting in Mammalian Cells

From early studies focused on the mammalian RIDD pathway it became clear that under acute instances of ER stress the number of mRNAs targeted to the RIDD pathway was significantly lower than the number observed in *Drosophila* cells (Hollien and Weissman, 2006; Hollien *et al.*, 2009). However, it was also clear that mammalian Ire1 is capable of broad mRNA cleavage when overexpressed or hyperactivated (Han *et al.*, 2009; So *et al.*, 2012). I set out to determine how mRNAs are prioritized to the RIDD pathway in mammalian cells. Based on both sequence and structural mutations to RIDD targets, I determined that in mammalian cells degraded mRNAs contain canonical Xbp1-like stem loops, which are required for cleavage by Ire1. However, I also show that an Xbp1-like stem loop is not sufficient to target mRNAs to RIDD, indicating that there must be additional levels of regulation in the RIDD pathway.

While there has been evidence that translation plays a role in the RIDD pathway in *Drosophila* cells (Gaddam *et al.*, 2013), this work was the first to demonstrate that inhibiting translation is required for acute induction of RIDD in mammalian cells. We show that translation attenuation, mediated by either Perk signaling or sequence elements within the mRNA, allows for cleavage of mammalian RIDD targets. Based on these results we hypothesize that attenuation of translation allows for stem loop formation within the mRNA transcript, which prioritizes these transcripts for rapid degradation by Ire1. As

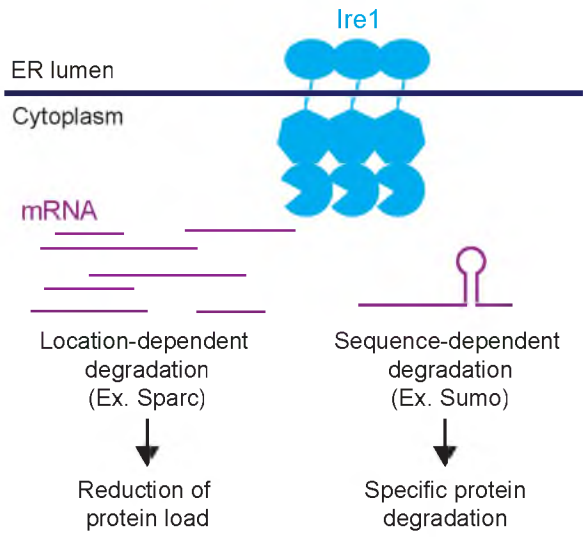

Figure 5.1 Models of RIDD function in *Drosophila.* Location-dependent and sequence-dependent mRNA degradation via RIDD, and possible downstream consequences of each, are shown.

noted earlier, during extended activation of Ire1, the requirements for stem loops and translation attenuation appear to be lost (Han *et al.*, 2009; So *et al.*, 2012). In these scenarios the mRNAs degraded by mammalian Ire1 resemble the mRNAs degraded in flies, in that they are enriched in mRNAs that encode proteins of the secretory pathway, but not for Xbp1-like stem loops. We propose a model in which mammalian Ire1 rapidly cleaves prioritized mRNAs with accessible stem loops, but also nonspecifically cleaves mRNAs localized to the ER. The latter form of cleavage occurs at a much lower rate than observed in Drosophila cells, and degradation of these mRNAs is measurable only at later time points. These distinctions in targeting may result in different functional outcomes for the cell with early degradation of specific targets being beneficial for the cell, while unremitting nonspecific cleavage of mRNAs localized to the ER may result in cell death through increased loss of proteins required to mediate the UPR.

Difference Between RIDD in Flies and Mammals

Although there is conservation of the RIDD pathway between flies and mammals, there are clear differences in target selection for Ire1 cleavage. In both flies and mammals, Ire1 is capable of nonspecific cleavage dependent on mRNA localization (location-dependent), as well as specific cleavage based on mRNA structure and sequence (sequence-dependent). The differences within these systems emerge in RIDD target prioritization. While location-dependent and sequence-dependent RIDD occur concurrently in flies (Hollien and Weissman, 2006; Moore *et al.*, 2013), longer periods of Ire1 activation are required to

observe location-dependent degradation in mammals, indicating that it may be a secondary effect of Ire1 activation (Han *et al.*, 2009).

The biochemical basis for the different activities of fly and mammalian Ire1 remains unknown. The protein sequences of the kinase and RNase domains of Ire1 from flies and humans are highly conserved (69% similarity) (Figure 5.2A); however *Drosophila melanogaster* Ire1 has an extended C-terminal domain that is unique to flies (Figure 5.2B). Based on amino acid composition, the C-terminal domain is expected to have a net positive charge. This raises the intriguing hypothesis that the positively charged c-terminal domain of Ire1 forms inter-molecular interactions with mRNAs, possibly resulting in increased retention of RNA in the vicinity of Ire1's RNase domain allowing for increased rates of location-dependent mRNA cleavage in *Drosophila* cells.

Based on the mechanistic differences of the RIDD pathway in these organisms it seems likely that there are also differences in their physiological functions. We hypothesize that the relatively rapid degradation of mRNAs encoding secretory proteins, characteristic of RIDD in flies, relieves stress on the ER by two mechanisms. First, degrading mRNAs decreases translation of proteins that would otherwise increase the folding load on the ER. Second, loss of mRNAs surrounding the ER frees up translational machinery as well as Sec61 channels allowing for faster production of the proteins upregulated by the UPR. Furthermore, mRNAs that are undergoing translation may be resistant to RIDD, and thus proteins that are necessary to carry out the UPR could avoid degradation via increased translation. This model does not exclude the possibility that degradation of specific mRNAs plays a role in the unfolded protein response,

A

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Fly_Ire1            EEKMLSFLQDVSDRVEKLQFHAEPLKSLEKNGRIVVLDDWNVHLDPMITDDLRKYRGYMG
Human_Ire1alpha     LEKQLQFFQDVSDRIEKESLDGPIVKQLERGGRAVVKMDWRENITVPLQTDLRKFRTYKG
Mouse_Ire1alpha     LEKQLQFFQDVSDRIEKEALDGPIVRQLERGGRAVVKMDWRENITVPLQTDLRKFRTYKG
                      * *.*:******:**  : .  :..**..** **  **. ::   :  ****:* * *

Fly_Ire1            ASVRDLLRALRNKKHHYHELTPAAQKMLGCIPHEFTNYWVDRFPQLISHAYHAFSICSNE
Human_Ire1alpha     GSVRDLLRAMRNKKHHYRELPAEVRETLGSLPDDFVCYFTSRFPHLLAHTYRAMELCSHE
Mouse_Ire1alpha     GSVRDLLRAMRNKKHHYRELPVEVQETLGSIPDDFVRYFTSRFPHLLSHTYQAMELCRHE
                    .********:*******.**.  ..: **.:* :*. *:..***:*:::*:*.*:.:* :*

Fly_Ire1            PIFKPYYSAGYLFTRPWYFDADDALFPMLMLDPKPLPKIGSPKKTPSPASSQAQQLKQRK
Human_Ire1alpha     RLFQPYY-------------------------------------------------------
Mouse_Ire1alpha     RLFQTYY-------------------------------------------------------
                     :*:.**

Fly_Ire1            GLYNFRKPNDELPIPGVGLQRNLELDGQSLEPDGKRDVFANFKFRRYSKPGNNRNYNGGH
Human_Ire1alpha     ----FHEPPEPQP---------------PVTPDAL-------------------------
Mouse_Ire1alpha     ----WHEPTEPQP---------------PVIPYAL-------------------------
                        :.:* :  *               .: * .

Fly_Ire1            KEAQDKEKYVIWTLPPSTQD
Human_Ire1alpha     --------------------
Mouse_Ire1alpha     --------------------
```

B

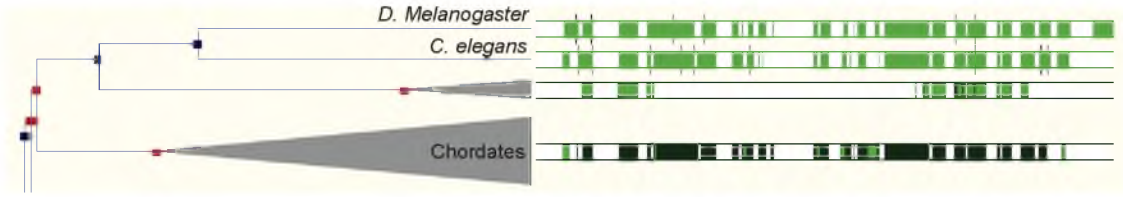

Figure 5.2 Ire1 sequence conservation in flies and mammals. (A) Amino acid alignment of the RNase domains of *Drosophila melanogaster* Ire1, *Homo sapiens* Ire1α, and *Mus musculus* Ire1α. The catalytic sites of the RNase domain are noted in blue, and the extended C-terminal tail of fly Ire1 is in red. * denotes conservation of identical amino acids in all three organisms. (:) or (.) denotes conservation of amino acids with similar chemical properties. (B) Ire1 protein conservation across multiple organisms. Green and black bars both represent protein alignment of Ire1 from various species. White blocks denote gaps in the alignment. The C-terminal tail is unique to flies and not found in any other organisms. Figure based on data from Ensembl (Cunningham *et al.*, 2015).

but rather that specific and nonspecific cleavage work in a coordinated manner.

We hypothesize that in mammalian cells the specific degradation of mRNAs at early times provides a different function. Although degradation of a small number of mRNAs could hypothetically downregulate translation of specifically hard to fold proteins, which alone can induce ER stress, the fact that the most consistent RIDD target in mammalian systems (Blos1) does not appear to enter the ER argues against this model (Scott *et al.*, 2012). Instead, we hypothesize that degradation of a specific target mRNA is the biologically relevant effect of RIDD in mammalian cells. The effects of long term RIDD, which results in both location-dependent and sequence-dependent mRNA degradation, remain unclear. In mammalian cell culture systems long term induction of RIDD appears to induce apoptosis, most likely through continuous degradation of mRNAs required for the UPR (Han *et al.*, 2009); however, mice lacking Xbp1, which results in hyperactivation of Ire1 and long-term RIDD activity, appear to have better outcomes than wild type mice in various disease models (So *et al.*, 2012), indicating that within an organism, cells may be able to compensate for the continuous loss of ER-localized mRNAs.

A Possible Role for RIDD of Blos1

In mammalian cells the only mRNA that has been shown to be degraded by RIDD in multiple systems and types of stress is Blos1 (Bright *et al.*, 2015). Thus we predicted that the ubiquitous degradation of Blos1 during ER stress may have functional consequences for the cell. Based on evidence that depletion of Blos1 by siRNA results in perinuclear accumulation of lysosomes (Pu *et al.*,

2015), we hypothesized that degradation of Blos1 mRNA during ER stress would result in the same phenotype. Here we show that lysosomes do accumulate in the perinuclear region of cells starting approximately 8hr after induction of ER stress and continuing through at least 18hr of stress. We also show that this accumulation occurs in an Ire1-dependent, Xpb1-independent manner. In the near future we plan to determine the specific contribution of Blos1 degradation to lysosome movement, as well as the additional components involved in this pathway and its functional consequences during ER stress.

Concluding Remarks

A cell's ability to respond appropriately to the changes in its environment contributes to both cell and organism fate. The proper function of the ER, as the hub of folding and quality control for secreted proteins, is essential for these responses. However, only within the last 20 years, have we started to discover the pathways of the UPR that sense and regulate the environment of the ER. The physiological importance the UPR is clear, but our understanding of how each pathway is regulated and fits into the global response to perturbations in ER function remains incomplete. These gaps in knowledge make it challenging to create targeted therapeutics to treat diseases in which the UPR is activated. In this work I have discovered the regulatory mechanisms of mRNAs targeting to the RIDD pathway in both flies and mammals, and propose a model in which mRNA degradation via RIDD promotes the increases in autophagy observed during ER stress. Through these insights and our continued work, we are connecting RIDD signaling to the overall response to ER stress.

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